SOBRE DOS COMPONENTES DE LA FISIOLOGIA MITOCONDRIAL: EL COMPLEJO DE LA NADH DESHIDROGENASA Y LA ATP SINTETASA

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ABREVIATURAS

ADP	Di-fosfato de Adenosina
ANS	8-anilino naftaleno 1-sulfonato
ATP-	Tri-fosfato de Adenosina
CMCD	Carboxi metil carbo di-imida
CTP	Tri-fosfato de Citidina
DCCD	Di-ciclo hexil carbo di-imida
D_2O	Oxido de Deuterio o Agua deuterada o Agua Pesada
ε-ATP	1-N^6-etenoadenosina 5'tri-fosfato
EDTA	Acido tetra acético Etilen-diamino
EPR	Resonancia paramagnética del electrón
FMN	Mononucleótido flavínico
GTP	Tri-fosfato de Guanosina
ITP	Tri-fosfato de Inosina
MMIQ	1-(p-methoxybenzyl)-6-7-methylene dioxyisoquinolina
NADH	Di-nucleótido de nicotinamida en su forma reducida
PITC	Fenil isotiocianato
PSM	Partículas submitocondriales
SHTP	6-mercaptopurina ribósido tri-fosfato
SDS	Dodecil sulfato de sodio
TMA-Cl	Cloruro de tetra-metil amonio
TNBS	Tri-nitrobenzen sulfonato
UTP	Tri-fosfato de Uridina

CAPITULO I

INTRODUCCION GENERAL

El sistema transductor de energía en las mitocondrias está formado por dos grandes complejos morfo-funcionales que son la cadena respiratoria y la unidad de síntesis de ATP. Estos dos sistemas han podido ser aislados como complejos multisubunitarios funcionales con una estructura muy complicada debido principalmente al número y naturaleza de los componentes que los integran. Corresponden a la cadena respiratoria los complejos I, III y IV; a la ATP sintetasa se le denomina complejo V. (1).

En el curso de esta introducción, se analizarán los complejos que llevan a cabo los procesos de la fosforilación oxidativa y el transporte de electrones y se enfatizarán aquellos puntos sobre los cuales versa el contenido de los trabajos que en esta tesis se conjuntan y en los cuales se encuentra información adicional.

A. LOS COMPLEJOS DE LA FOSFORILACION OXIDATIVA

1. COMPLEJO DE LA NADH-Q REDUCTASA O COMPLEJO I.

Constituye el componente mas grande de la membrana mitocondrial interna con un peso molecular de aproximadamente medio millón. A este complejo lo forman 16 polipéptidos según el análisis en geles de SDS, 23-36 nmoles de azufre lábil a ácido, 23-26 mg átomo de Fe no hemo por mg de proteína, 1.4-1.5 nmolas de FMN, 4.2-4.5 nmolas de ubiquinona y 0.22 mg de lípido por mg de proteína (1). Cuando el complejo I se trata con agentes caotrópicos se producen una fracción hidrofílica y una hidrofóbica lo cual no implica que se encuentren en esa forma en la proteína nativa. La primera contiene una flavoproteína Fe-S, un FMN, una proteína con Fe-S y 4 centros de Fe-S(2).

La flavoproteína contiene a su vez dos subunidades. Se sabe por estudios realizados con marcadores de superficie que casi toda la proteína está inmersa en la membrana, aunque hay una "hendidura" hidrofílica en la cual se localiza el FMN que podría dar cabida al NADH. La flavoproteína parece contener también 2 centros de Fe-S binucleares y a juzgar por las señales en EPR, ambos centros son reducibles por NADH y ditionita (3). La flavina no está covalentemente unida a la apoproteína.

La fracción hidrofóbica resultante del tratamiento con agente caotrópico parece contener centros Fe-S tetranucleares. Esta fracción con un peso molecular de 33 000 se marca con Iodo en presencia de lactoperoxidasa sólo en el Complejo I aislado, y no cuando está incorporado en liposomas, lo que ha sugerido su intervención en la conducción de protones en el Complejo I (4).

El ferricianuro sirve como un oxidante para la deshidrogenasa tanto pura como adosada en las PSM. En mitocondrias intactas, la reducción del ferricianuro por sustratos dependientes de NAD^+, se inhibe por rotenona (5).

El mecanismo por el cual una transferencia de electrones se acompaña de una eyección de H^+ al exterior mediado por el complejo I reviste características especiales, dado que además de estas transferencias, en este segmento de la cadena transportadora de electrones se localiza el llamado sitio I de acoplamiento.

Para explicar los mecanismos de la translocación de protones dependientes de reacciones redox, se han establecido dos modelos de acoplamiento (6): el directo y el indirecto. El primero está representado por el concepto de asa redox de Mitchell (7) y por la idea mas general de las reacciones vectoriales y la translocación de grupo; en este mecanismo el acarreador redox es

también un acarreador de H^+ al cual el H^+ se une covalentemente cuando es translocado junto con el electrón. Este proceso es electroneutro y lo llevarían a cabo moléculas como la ubiquinona; posteriormente el electrón fluiría de regreso a través de un acarreador de electrones del tipo de los citocromos. El mecanismo indirecto es básicamente igual al mecanismo directo pero difiere en que el H^+ que se transfiere no está unido a la molécula redox, sino que se fija a otro sitio que a su vez está ligado indirectamente a la reacción de óxido-reducción. Tal parece que en la naturaleza ambas formas de acoplamiento se encuentran expresadas casi igualmente en sistemas tanto mitocondriales como en cloroplastos.

Un hallazgo importante para dilucidar y disecar los componentes de la cadena respiratoria lo constituyen los inhibidores específicos.

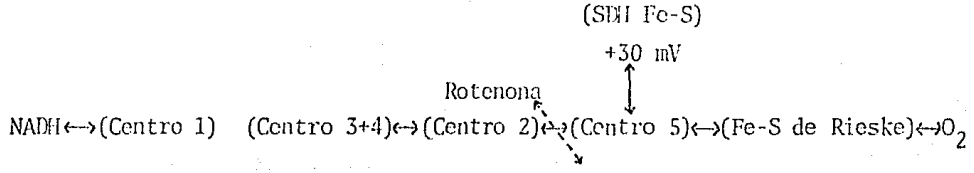
Para el componente ATPsintetasa (Complejo V), existe una variedad extensa de compuestos capaces de inhibir la actividad catalítica: antibióticos, análogos de nucleótidos, compuestos con grupos reactivos específicos, etc. Gracias a su diversidad y por tanto a sus diferentes mecanismos de acción, ha sido posible predecir, sugerir y postular la existencia de grupos importantes en la catálisis y/o su posible papel en los eventos del proceso.

Lo mismo ha sucedido con los complejos de la cadena respiratoria. Gracias a los efectos específicos de inhibidores tales como la rotenona (8), el amital (9), la piericidina (10), la antimicina (11) y el cianuro (12), fue posible conocer a los componentes de la cadena respiratoria y comenzaron a inferirse los mecanismos del proceso de transferencia de electrones, y el arreglo secuencial de sus componentes.

La rotenona y el amital fueron inhibidores propuestos para la flavoproteína NADH deshidrogenasa (8) en base a estudios que incluían titulaciones

de los inhibidores y sus efectos sobre el consumo de oxígeno mitocondrial, la oxidasa del NADH, la relación P:O, la respiración, la hidrólisis de ATP y el intercambio ATP-^{32}P. Las actividades dependientes de la oxidación de sustratos fueron probadas con piruvato, malato, NADH, succinato, α-cetoglutarato y DL-β-OH butirato. Quedó establecido que el amital, la rotenona y la piericidina eran inhibidores de la actividad de la NADH deshidrogenasa, por lo que el flujo de electrones entre este complejo y la ubiquinona quedaba bloqueado (8,10).

Sin embargo se desconoce el sitio preciso de acción de los inhibidores sobre el llamado sitio I de acoplamiento. De una manera funcional se ha podido detectar el posible sitio de inhibición de la rotenona a través de la señal que dan en EPR los centros Fe-S del segmento NADH DH-Co Q. El esquema que se ha propuesto para dichos centros en PSM de corazón de res es el siguiente (13):



Tal vez debido al gran número de sitios sensibles a inhibidores que puede presentar el complejo NADH deshidrogenasa, es que estos pueden incluir variaciones en su modo de acción, por ejemplo se requieren de 24-28 nmolas de

rotenona por mg de proteína mitocondrial para inhibir el transporte de electrones dependientes de NADH. En cambio para el amital, la antimicina A y la oligomicina se requieren concentraciones más elevadas. Por otro lado mientras la rotenona no inhibe la reacción del cambio ATP-P^{32} el amital, inhibidor también del sitio I, sí lo hace. Es claro que estas diferencias pueden deberse a que no sólo el complejo I sino otros complejos pueden ser receptores de un mismo inhibidor, lo que dependería de la estructura del inhibidor. En el caso de la rotenona y la antimicina se trata de compuestos fundamentalmente hidrofóbicos, aunque contienen algunos sustituyentes polares en el armazón heterocíclico, ambas características probablemente sean importantes para permitir su interacción con otros componentes de la membrana.

El MMIQ es un compuesto que tiene una estructura hidrofóbica y contiene grupos OCH_3, N y $CH_2\begin{matrix}O\\O\end{matrix}$; considerando estas características y las propiedades que comparte con la octilguanidina como la de poseer carga positiva a pH 7 (14), hicieron del MMIQ un compuesto interesante en cuanto a la acción que podría tener sobre la fosforilación oxidativa. En la Sec. A, Cap. II se presentan las evidencias que llevan a la conclusión de que el MMIQ interacciona con el segmento NADH-CoQ, lo que resulta en una inhibición del flujo de electrones.

COENZIMA Q_{10} O UBIQUINONA

Este es un derivado de la quinona con una cadena lateral isoprenoide de 10 unidades. Es liposoluble y se ha postulado como un translocador de protones en base a estudios cinéticos y de reconstitución (2). Su papel es fundamental para establecer la asociación entre las deshidrogenasas (succínica y

del NADH) y el complejo de citocromos bc_1 (8).

Para la teoría quimiosmótica que presupone la existencia de componentes transportadores de H^+, la ubiquinona es un candidato para cumplir esta función, movilizando protones desde la matriz mitocondrial hasta el citosol. Esta suposición implicaría la existencia de una poza de ubiquinona que difunda de la membrana a los sitios de las deshidrogenasas y los citocromos, posibilidad que no ha sido descartada dada la abundancia de Co Q_{10} en los complejos I y III (17).

2. COMPLEJO DE LA UBIQUINOL CITOCROMO C OXIREDUCTASA O COMPLEJO III.

También es llamado complejo bc_1, y está constituido por 7 u 8 subunidades polipeptídicas en las que se hallan integrados cinco centros de óxido-reducción, cuatro de ellos son: dos o tres tipos de citocromos b, una proteína con Fe-S (el fierro en grupo no hemo) llamada proteína de Rieske, y un citocromo c denominado c; contiene también una proteína que contiene Q_{10} y fija antimicina y una proteína central (18,19). Las subunidades pueden presentar estequiometrías de uno o dos y sus pesos moleculares están entre 52,000 y 9,000 daltones (20,21), sin embargo no se le ha asociado una función a cada una, aunque se sabe que las subunidades III y IV, denominadas así según su posición en el patrón electroforético de geles en SDS, contienen centros redox (22). El citocromo c_1, es una proteína que pesa 43 600 y está integrado al complejo III ya que solo es sensible a la acción de la tripsina cuando se le aisla (8). Se le considera una proteína integral de membrana, cuya extracción se facilita con la presencia de agentes reductores (23).

Topológicamente se ha propuesto que el complejo se encuentra en forma dimérica y aunque ésto se sabe a partir del analisis de la imagen computarizada del microscopio electrónico de los cristales del complejo (22), tal parece que ésta puede ser la forma que se encuentra in situ; es probable que el dímero se encuentre atravesando el grosor de la membrana y que por consiguiente tenga zonas hidrofóbicas y zonas hidrofílicas. Se ha tratado de encontrar la distribución de las subunidades dentro de estas zonas mediante el uso de anticuerpos, de reactivos hidrofóbicos y de marcadores de superficie, pero no se tienen datos claros hasta ahora.

3. COMPLEJO DE LA CITOCROMO C OXIDASA A-A_3 O COMPLEJO IV.

Se ha encontrado que el complejo de la citocromo oxidasa, presenta gran semejanza con el complejo b-c_1. El complejo a-a_3 contiene 7 subunidades polipeptídicas, cuyos pesos moleculares se hallan en el rango entre 35000 y 50000 y su estequiometría aparentemente es 1. El complejo pesa aproximadamente 140 000. Es probable que este complejo se halle en forma dimérica (22) y que contenga una región hidrofóbica y una hidrofílica (25). El complejo tiene 2 grupos hemo (a y a_3) y 2 átomos de cobre ($Cu_A^{++(+)}$ y $Cu_B^{++(+)}$) por monómero. El grupo hemo a_3 se encuentra asociado tanto física como funcionalmente con el $Cu_B^{++(+)}$; los dos átomos metálicos forman un centro binuclear que cataliza la oxidación de O_2 a H_2O. Se ha demostrado que la citocromo oxidasa cataliza la translocación de H^+ asociada a la transferencia de electrones (26).

La reconstrucción de imágenes de microscopía electrónica ha mostrado que

la citocromo oxidasa es una proteína en forma de Y, dos terceras partes de la cual están embebidas en la membrana mitocondrial interna. El resto está expuesto al lado citoplásmico de la membrana (27).

La intensa búsqueda del arreglo de los polipéptidos en el complejo y la localización en ellos de los sitios de unión del citocromo c y de los sitios responsables de la translocación de protones, hacen de la citocromo oxidasa el candidato mas probable de los 4 complejos de la cadena respiratoria por ser conocido detalladamente.

4. ATP SINTETASA O COMPLEJO V.

El V complejo en el sistema transductor mitocondrial de energía es la también llamada ATPasa translocadora de protones, OS-ATPasa, DCCD-ATPasa, o complejo F_1-F_o (28). Esta enzima está constituida por dos porciones localizadas en diferentes medios: la porción soluble en H_2O llamada F_1 y la F_o que integra la porción hidrofóbica en la membrana y que es altamente hidrofóbica.

El sector soluble de la ATPasa pesa 360 000 daltones y está constituído por 5 diferentes subunidades: $\alpha,\beta,\gamma,\delta,\varepsilon$. Sin embargo la estequiometría de las subunidades está sujeta a discusión y se ha propuesto que puede ser de 2:2:1:1:1 o de 3:3:1:1:1; su topología y función no están resueltas, aunque hay datos que sugieren que la subunidad β contiene el sitio catalítico (29,30); la subunidad α puede tener una función reguladora (31); las subunidades γ,δ y ε parecen tener contacto físico con el canal de H^+ y quizás regulen el flujo

de H^+ desde la F_o hasta el sitio de la catálisis (32).

La porción membranal ó F_o, contiene varias proteínas entre las que se han identificado la F_6, la proteína que confiere sensibilidad a la oligomicina y otra proteína de 10 000 daltones que es un proteolípido que fija DCCD (33,34). Se encuentra también una proteína inhibidora de 10 000 daltones (35) que se tratará detalladamente más adelante.

El funcionamiento de este complejo como ATP sintetasa se ha tratado de explicar mediante 2 diferentes modelos principalmente: el conformacional propuesto por Boyer (36) y el quimiosmótico propuesto por Mitchell. Los postulados básicos de la Teoría quimiosmótica parecen encajar en un esquema en que la fuerza protomotriz es la que lleva a la formación de ATP, sin embargo el mecanismo fino molecular que supone la formación del enlace anhidrido entre el ADP y el P_i permanece como materia de continua búsqueda y controversia.

A la ATP sintetasa se le conocen las siguientes actividades:

a) Actividad hidrolítica. La pueden llevar a cabo tanto la OS ATPasa como la F_1 soluble. Los sustratos hidrolizables pueden ser varios nucleótidos trifosfatados: ITP, GTP, UTP, CTP, SHTP, $\equiv$ ATP y arilázido aminopropionil ATP (28) Según las condiciones de ensayo la Km para el ATP está entre 0.2 - 1.25 mM, el ADP es un inhibidor competitivo con una K_i = 30-100 µM (37). La reacción es inhibida por aurovertina, la proteína inhibidora, el ortofosfato y el arsenato, y es estimulada por el bicarbonato y el bisulfito (38).

b) Síntesis de ATP. Esta sólo es catalizada por el complejo F_1 - F_o ya que debe establecerse un flujo de H^+ generado por el potencial electroquímico producido por la cadena respiratoria. La reacción se inhibe por DCCD, oligomicina, aurovertina, y desacoplantes del transporte de electrones (39).

La estequiometría propuesta para ATP formado por H^+ transportados puede ser 1:2 ó 1:3 (40, 41). Debido a la diferencia de comportamiento cinético y sensibilidad a inhibidores se ha sugerido la posibilidad de que existan en la enzima diferentes sitios para la hidrólisis y la síntesis del ATP (42).

c) Translocación de H^+ ligada a la hidrólisis de ATP. Este proceso sólo es llevado a cabo por el complejo F_1 F_o embebido en una membrana, el movimiento vectorial de los protones es fundamentalmente promovido por el sector F_o del complejo, permitiéndole que funcione como una bomba de protones, tal actividad es inhibida por oligomicina, DCCD, venturicidina, etc. (39).

d) Reacciones de Intercambio. Se ha encontrado que la ATP sintetasa y la F_1 soluble catalizan varias reacciones de intercambio dependientes de energía que son importantes ya que constituyen indicios de los eventos químicos involucrados en la catálisis y pueden llevar a considerar o rechazar mecanismos propuestos. Las reacciones de intercambio son:

1) Reacción de intercambio ATP-^{32}P. Como requisitos para que esta reacción se realice están la presencia de una membrana y el acoplamiento a ésta del complejo F_1F_o (43). El fosfato que se transfiere durante la reacción es el P_i y del ATP.

$$ATP + {}^{32}P_i \rightleftharpoons \gamma\text{-}{}^{32}P + P_i$$

Esta reacción parcial es sensible a los inhibidores de la hidrólisis, a desacoplantes, a DCCD y a oligomicina.

2) Reacción de intercambio ADP-ATP. Parece que los requerimientos y los inhibidores para esta reacción son los mismos que para la del recambio ATP-^{32}P (44). Quizás sea la reacción menos estudiada de todas las que integran el recambio.

$$^{14}C - ADP + ATP \leftrightharpoons ^{14}C - ATP + ADP$$

3) Reacción de intercambio ATP-HOH. En esta reacción el oxígeno del agua se incorpora al fosfato terminal γ del ATP vía el sistema intacto de ATPasa unido a la membrana.

$$ATP + H_2\,^{18}O \rightleftharpoons ATP(^{18}O) + H_2O$$

La reacción es sensible a desacoplantes, oligomicina, DCCD y a los inhibidores de la F_1, y también es sensible a arsenato (45).

4) Reacción de intercambio P-HOH. Esta reacción es sensible sólo parcialmente a los desacoplantes y totalmente inhibida por oligomicina, por lo que Boyer (46) ha propuesto que requiere una fuente de energía no sensible a los desacoplantes y ha demostrado que ésta puede provenir de la hidrólisis de ATP. La reacción también es sensible a otros inhibidores como DCCD y los de la F_1.

$$P_i + H_2\,^{18}O \rightleftharpoons Pi(^{18}O) + H_2O$$

B. LA EXPRESION DE LOS COMPLEJOS.

1. EFECTO DEL K^+

Gracias al empleo de efectores de diferente naturaleza como herramientas en el estudio de la estructura y la fisiología mitocondrial, ha podido avanzarse en su conocimiento. Podemos nombrar como ejemplos de tales efectores: los ionóforos (47), compuestos hidrofóbidos (48,49), aniones (50,51) y cationes (52,53). Entre estos últimos se ha explorado la acción de los cationes monovalentes como el K^+.

El problema de los movimientos de K^+ en la mitocondria aislada es muy interesante, ya que las concentraciones de K^+ intramitocondriales de este catión están entre 150 y 250 mM, lo cual significa que hay un gradiente de concentración contra el cual se mantiene el K^+ interno y por lo tanto debería ser posible postular mecanismos de transporte correspondientes.

Por otra parte, los efectos producidos por el K^+ sobre la mitocondria se han observado en diferentes estructuras y con variedad cualitativa: van desde la estimulación de la fosforilación oxidativa (54,55), a un efecto desacoplante (56) y a no producir ningún efecto (57).

Sin embargo, decir que la fosforilación oxidativa se ve afectada por el K^+ implica la existencia de un gran número de sitios sensibles a la acción del catión, ya sea sobre las enzimas que llevan a cabo el transporte de electrones o sobre la ATPasa misma, los cuales serían efectos directos sobre la fosforilación oxidativa, pero también podrían observarse efectos indirectos, como puede suceder en el caso de que el transporte de K^+. Tuena de Gómez-P. y Gómez-Puyou han descrito una técnica: incuban las mitocondrias con Na^+,

EDTA, fosfato, y sustratos oxidables durante 3 min, así los valores de K^+ disminuyen a 10 nmolas por mg de proteína (59).

En mitocondrias depletadas de K^+ hasta una concentración de 15 mM, el K^+ añadido en los medios de incubación a-menta la relación P:O y la respiración (56), además el efecto del K^+ es mimetizado por otros cationes monovalentes como Li^+, Rb^+, Cs^+ y el $TMAC1^+$; la acción de estos cationes es al parecer de localización interna, ya que estos experimentos se realizaron intercambiando K^+ exógeno en presencia de valinomicina. El K^+ también aumenta el control respiratorio, disminuyendo la velocidad de consumo de oxígeno en el estado 4; es posible que este efecto se deba a un incremento en la entrada de sustratos oxidables.

Para superar los problemas de efectos de permeabilidad a sustratos como NADH, succinato, etc., se han desarrollado estrategias como determinar los efectos del K^+ en partículas submitocondriales (58) o F_1 aislada (59).

Sin embargo en partículas submitocondriales se han obtenido resultados contradictorios: dos grupos han encontrado que la velocidad de oxidación de NADH o del β-OH butirato se ve estimulada por K^+, sin embargo, esto no ocurre cuando el sustrato oxidable es succinato (58,60). De estos resultados se llegó a la conclusión de que el efecto primario del K^+ se localiza en la cadena respiratoria y muy probablemente a un nivel entre la NADH deshidrogenasa y la ubiquinona. Por otra parte Lee y Ernster (61) encontraron fosforilación oxidativa en partículas submitocondriales depletadas de K^+, mientras Papa y cols. (62) hallaron inhibida la fosforilación oxidativa a altas concentraciones de K^+.

A diferencia de lo que sucede a nivel de cadena respiratoria con la estimulación por K^+, el efecto en la ATPasa soluble es altamente específico, ya que otros cationes monovalentes no estimulan la actividad hidrolítica de la F_1 aislada (48,63).

Con respecto al transporte de adenín nucleótidos a través de la membrana mitocondrial, se ha encontrado que el K^+ produce un efecto de facilitación sobre el intercambio de adenín nucleótidos (64,65).

Respecto al influjo de K^+ a la mitocondria, se sabe que la entrada es óptima en condiciones en las que hay transporte de electrones, EDTA y fosfato en el medio. Estos, al igual que la presencia de otros cationes monovalentes (66, 67), son factores que influyen tanto en la magnitud como en la direccionalidad de los movimientos del catión a través de la membrana interna, ya que también el eflujo de K^+ es sensible a las condiciones mencionadas. En lo que respecta a la acción de otros cationes monovalentes como el Na^+, parece ser que ambos, Na y K^+, son transportados por sistemas diferentes, ya que mientras el transporte de K^+ es inhibido por Tl^+ no lo es el de Na^+ (67).

Tal parece que con el establecimiento del potencial electroquímico bajo condiciones de energización, hay una entrada electroforética de K^+ hacia la matriz mitocondrial, mientras que la salida de K^+ desde la misma podría deberse a los niveles de NADPH y Pi (68). El intercambio puede inhibirse por desacoplantes de la fosforilación oxidativa.

2. EFECTO DE SOLVENTES: EL OXIDO DE DEUTERIO Y EL METANOL.

Las proteínas de membrana pueden estar influenciadas ya sea en forma sutil o bien dramática, por el medio lipídico en que se encuentran incluídas parcial

o totalmente. Esta influencia puede hacerse patente en su estructura y de ahí, repercutir en su funcionamiento (69). La bicapa lipídica posee características especiales definidas y diferentes a las de un medio acuoso. (70).

La ATPasa mitocondrial constituye un caso muy claro del cambio de comportamiento de una enzima de membrana cuando se extrae de su ambiente en la bicapa. Cuando la enzima está en forma particulada, su Km para el ATP es de 300 μM, y en forma soluble de F_1, la Km es de 100 uM (37,40), además hay evidencias de que el ATP tiene más afinidad por los sitios de alta afinidad cuando la enzima está unida a la membrana.

La temperatura es otro de los factores que afectan de manera diferencial a la ATPasa: la F_1 es inactivada por frío, mientras que la enzima particulada no, lo es, excepto en condiciones experimentales especiales (71,72). En la F_1 de mitocondrias de levadura se han obtenido datos que sugieren que las bajas temperaturas promueven la disociación de las subunidades, principalmente, la α del resto de la enzima, a través del análisis electroforético de los productos de la disociación producida por el frío (73).

Es interesante también que las subunidades α y β de la ATPasa, (la última de las cuales se sabe que forma parte del sitio catalítico)(30, 39), no son accesibles al marcaje con un reactivo radiactivo cuando la enzima está en forma soluble, y sí quedan marcadas cuando la enzima se encuentra en partículas submitocondriales (24).

Algunos autores basándose en hechos como los mencionados anteriormente han sugerido que la ATPasa membranal puede encontrarse embebida casi totalmente en la membrana, pero que en ciertas condiciones puede emerger de la superficie de ésta (25). Sin embargo, mediante la técnica de tinción negativa por microsco-

pía electrónica, se ha identificado a la F_1 en las llamadas partículas de Fernández-Morán (76) como botones que sobresalen de la membrana interna mitocondrial cuando ésta se invierte (77). Estas interpretaciones han sido objetadas por argumentos basados en que la metodología que implica la obtención de estas microfotografías puede introducir artificios, ya que el choque hipotónico que se aplica para el rompimiento de las membranas puede afectar la posición de la ATPasa.

Es por esto que es conveniente explorar el efecto de solventes de propiedades muy diferentes a las del agua y aproximadas a las de la membrana.

La intervención del solvente, cuanto éste es un solvente isotópico como el agua deuterada puede darse a diferentes niveles de la estructura y función de una enzima. Estos efectos se producen merced a la diferencia entre las propiedades químicas entre el H_2O y el D_2O, como pueden observarse en la siguiente tabla:

Propiedad	H_2O	D_2O
Punto de ebullición	100°C	101.42°C
Punto de congelación	0C°	3.81°C
Viscosidad	1.0005 centipoise	1.25 centipoise
Densidad[1]	0.9982 g/cc	1.1056 g/cc
Volumen molar	18.047 cc/mol	18.117 cc/mol
Constante dieléctrica[2]	78.54	78.93
Constante de ionización[2]	1.008×10^{-14}	1.95×10^{-15}

[1] A 20°C

[2] A 25°C

Concretamente, los efectos de un solvente como el agua deuterada pueden ser (78):

a) El agua puede actuar como un reactivo en el proceso de la catálisis enzimática, a ésto se le ha llamado efecto isotópico y puede ser primario o secundario.

b) El isótopo queda incorporado en el sitio activo de la enzima a través de reacciones de recambio.

c) El isótopo induce efectos estructurales en la arquitectura de la enzima, por intercambio con el H^+ involucrado en el establecimiento de puentes de H.

d) El isótopo al recambiarse produce alteraciones en las uniones de la capa primaria de solvatación de la enzima.

e) El isótopo al recambiarse produce cambios en los puentes de unión en regiones muy hidrofílicas de las proteínas y que pueden o no estar situados lejanamente del sitio de reacción.

f) El compuesto isotópico según sus características de viscosidad, densidad, etc., puede producir diferencias en la velocidad de las reacciones que constituyen la catálisis, según se hallen involucrados los fenómenos de difusión y movilidad en el contexto de funcionamiento de la enzima.

A partir de estos posibles efectos, resulta razonable pensar que la deuteración de los sitios con puentes de hidrógeno, puede inducir modificaciones en diferente grado sobre la arquitectura y los cambios conformacionales de la enzima. La magnitud de las modificaciones puede ser pequeña y no detectable como tal, pero traducirse en un efecto notorio en la velocidad de la catálisis.

Un ejemplo muy claro de este tipo de efectos en una enzima de tipo soluble es la sintetasa del formil-tetrahidrofolato (79). Esta enzima en su forma activa es un tetrámero que se forma a partir de la agregación de cuatro monómeros a través de la activación de cada uno de ellos con K^+ o Cs^+. El efecto que tiene el óxido de deuterio en este sistema es un incremento en la velocidad y en la constante de equilibrio de la formación del tetrámero. A un nivel mas fino se ha encontrado que el efecto isotópico se debe a la formación del complejo monómero-catión y no al paso de la combinación de un monómero con el siguiente.

En el caso de una proteína de membrana que ha sido estudiada en el agua pesada, se encuentra la ATPasa mitocondrial (80). De este estudio se ha desprendido que aparentemente el agua deuterada confiere a la enzima una cierta estabilidad a la desnaturalización por frío, a través del fortalecimiento de las interacciones hidrofóbicas entre las subunidades que conforman a la ATPasa, ya que de la disociación por frío es posible obtener disgregadas las subunidades

(81). Además, la actividad de hidrólisis de ATP disminuye con la incubación de la enzima en D_2O, lo cual ha sido interpretado como una posible rigidez de la estructura de la proteína.

Por los antecedentes mencionados es evidente que la ATPsintetasa resulta un excelente caso por ser estudiado, ya que sus propiedades como enzima unida a membrana difieren de las que posee como enzima soluble en medios acuosos, su posición en la membrana y las fuerzas que mantienen estructurada su arquitectura multisubunitaria, así como sus efectos en la catálisis, fueron las interrogantes que nos planteamos en la Sec. A., Cap. III, en la cual presentamos la información obtenida que señala la posibilidad de que la F_1 se encuentra embebida en la membrana, así como el papel que desempeñan las interacciones de tipo hidrofóbico entre las subunidades de la F_1 y el comportamiento cinético de la enzima expuesta a la acción del HCO_3^-, la octilguanidina y el K^+. Todo esto utilizando como herramienta al solvente mas parecido al H_2O, pero con características que lo hacen diferente: el D_2O.

Otro solvente explorado con el fin de ahondar en el mecanismo de reacción de la ATPasa, ha sido el metanol; a través del análisis de su influencia en el comportamiento cinético y termodinámico de la enzima. Con el metanol al 20% (v/v H_2O) (82) se observó un efecto estimulatorio de la hidrólisis de ATP e inhibitorio de la actividad de ATPsintetasa, aunque la energía de activación de la enzima en metanol, aumenta para ambas reacciones. Una explicación a estas observaciones es que el metanol interfiere con la liberación del ADP del sitio de la catálisis y dado que éste puede ser el paso limitante de la serie de reacciones de hidrólisis de ATP, el metanol puede facilitar la salida del ADP (83).

Otros autores utilizando la F_1 aislada de mitocondrias de levadura (84)

han encontrado que el metanol inhibe los sitios de unión de la aurovertina en la F_1 y también produce una activación de la actividad de ATPasa, lo cual han interpretado como una acción de este solvente sobre los sitios reguladores. A excepción de la interpretación, los datos de este grupo encajarían en el esquema de Harris (83), ya que es sabido que la aurovertina se fija a la subunidad β que se considera como parte (?) del sitio activo.

El metanol se escogió para explorar la cinética de la ATPasa, ya que es un efector que por sus propiedades físicas puede repercutir en las interacciones hidrofóbicas que intervienen en la actividad de la enzima ATP sintetasa. La Sec. B, Cap. III, hace énfasis en la cinética bifásica de hidrólisis de ATP y los factores como la octilguanidina y la temperatura que son capaces de modificar la actividad. Con este trabajo se sugiere la posibilidad de que el metanol al modificar el patrón de velocidad de hidrólisis en la segunda fase, lo haga a través de la inducción de una transición en la conformación de la F_1 requerida para el funcionamiento del ADP como inhibidor.

3. CAMBIOS CONFORMACIONALES.

Como fue indicado con anterioridad, una posible causa para explicar las diferencias entre la F_1 soluble y la particulada, puede deberse a la diferente conformación adquirida por la proteína dentro y fuera de la membrana, y a las subsecuentes modificaciones conformacionales durante la catálisis en diferentes medios.

En relación con los cambios conformacionales que puede sufrir la ATPasa durante la catálisis hay acuerdo en que existen y aunque se han podido detectar tanto en ATPasa unida a membrana (85,86) como en la F_1 (87), su interpre-

tación y participación en la serie de eventos que integran la catálisis y su relación con las reacciones de transferencia de electrones no están claras (40).

Las formas en que se han detectado cambios conformacionales en la ATP sintetasa son: el recambio de H con agua tritiada (88) el uso de reactivos blanco como el TNBS (89), el uso de reactivos entrecruzadores de grupos ditiol (90) y el empleo de moléculas sonda fluorescentes como la aurovertina y la fluoresceína (85,91). Pese a lo variado de la metodología, todas estas técnicas se basan en la exposición a los reactivos de grupos de la enzima en las condiciones experimentales que incluyen presencia de sustratos o efectores de la actividad de ATPasa como ATP, ADP, Pi, succinato, gradiente de pH, etc.

Los sustratos de la actividad de la ATPasa, ATP, ADP y Pi, tienen un sitio de unión en la enzima. Se han encontrado varios sitios de unión para nucleótidos ADP y ATP a partir de las siguientes observaciones (92):

a) Por la actividad de hidrólisis y síntesis de ATP, la enzima debe tener al menos un sitio de unión para ADP y otro para ATP.

b) El ADP es un inhibidor competitivo débil de la actividad de ATPasa.

c) Se han encontrado ADP y ATP radiactivos unidos a la enzima.

d) Se han encontrado unidos a la F_1 5 molas de nucleótido por mol de enzima. Hay desacuerdo para determinar cuantas de las moléculas son de ADP y cuantas de ATP, y de ellas cuales están firme o débilmente unidas.

e) Hay un retardo inicial de la hidrólisis cuando hay Mg^{++} y su duración depende de la concentración de ATP.

Se ha considerado que en los sitios de unión los nucleótidos pueden cumplir tres funciones: estructural, catalítica y reguladora (40,93,94) y aunque hay

toda una serie de datos incongruentes, el tema reviste la mayor importancia para dilucidar el mecanismo de acción de la enzima.

Para el fosfato, otro de los sustratos de las reacciones catalizadas por la ATP sintetasa, se han encontrado dos sitios de unión, uno de alta afinidad y uno de baja afinidad. El primero, con una constante de disociación de 80μ M a pH 7.5 y cuya unión depende de pH, une reversiblemente P_i (95,96). Acerca de la intervención de este sitio en la catálisis, Lauquin y cols. (97) han encontrado que el sitio al cual se une el P_i con una alta afinidad en la F_1 soluble, está en la subunidad β , guardándose al parecer una estequiometría de 1:1 entre este sitio y la enzima.

4. LA PROTEINA INHIBIDORA

Compuestos como la aurovertina, la octilguanidina y algunos cationes y aniones como los mencionados, pueden tener también sitios de unión en la F_1, y algunos pueden producir un efecto inhibitorio.

Sin embargo el inhibidor mas interesante de la ATPasa es el suyo natural: es una proteína que pesa 10 000 daltones y que fue descubierta por Pullman y Monroy (35) hace quince años, abriendo nuevas perspectivas en el conocimiento de los mecanismos de catálisis y regulación de la enzima.

Actualmente se sabe que la proteína inhibidora es una entidad común a todas las ATPasas de protones en las que se ha buscado: en mitocondrias de corazón de res (35,98), hígado de rata (99,100), levadura (101,102); en estos organismos el inhibidor es una proteína adicional a la F_1, pero en ATPasas de protones como las de las bacterias E. coli (102), A. faecalis (104) y M. lysodeikticus (105), el inhibidor constituye la subunidad ε de la ATPasa y lo mismo sucede con el inhibidor de la ATPasa de cloroplastos (106). Todos

han sido purificados y los pesos moleculares de los inhibidores de estas especies están entre 6000 y 16000 daltones.

El inhibidor de la ATPasa mitocondrial de corazón es el que mas se ha estudiado y por composición de aminoácidos, su peso molecular es de 10 000 daltones (107), aunque se han obtenido preparaciones de 6800 y 15000 daltones. También se ha descrito que el inhibidor tiende a asociarse en trímeros y tetrámeros y aún hexámeros (108). La composición de aminoácidos ha revelado también una gran cantidad de residuos de lisina y ácido glutámico principalmente, sin embargo aunque se le considera proteína básica su pH isoeléctrico es de 7.6 (109).

En cuanto a sus propiedades químicas, la proteína inhibidora de mitocondrias de corazón de res es estable al calor, a la acción de los álcalis, de ácidos, del frío y es específico para H^+ ATPasas ya que no inhibe a otro tipo de enzimas, aunque dé reactividad funcional con ATPasas de especies diferentes (35).

Son muy interesantes los requerimientos para la fijación de la proteína inhibidora a la F_1 soluble o particulada y al complejo V. El pH óptimo para conseguir la interacción es de 6.5 (35,98); Hatefi sugiere que a phs entre 6.8 y 7.0 hay residuos de aminoácidos del inhibidor con valores de pK neutros que participan en tal interacción (110). Otro de los requerimientos es la presencia de un catión divalente como Mn^{2+}, Ca^{2+}, Fe^{2+}, Mg^{2+}, Cu^{2+} Co^{2+} y Zn^{2+} (98). La hidrólisis de un nucleótido trifosfatado también es necesaria para la unión F_1-inhibidor y el ATP, ITP, UTP, GTP y CTP son eficaces para tal efecto (111).

Se cree que el papel que desempeña el nucleótido no es estructural, sino

que es la conformación transitoria que adquiere la enzima durante la hidrólisis del nucleótido, la que permite que el inhibidor se fije (112). La Kd calculada en F_1 soluble es 10^{-8}M (111).

Acerca del sitio de interacción del inhibidor con la ATPasa, el dato mas exacto de que disponemos es el logrado por Klein y cols. (113) en que el inhibidor natural de corazón marcado con un reactivo radiactivo de entrecruce, ^{14}C PITC, interacciona con la subunidad β de la F_1, según el análisis electroforético de los complejos inhibidor-F_1. Esta es la evidencia de interacción estructural, sin embargo también se dispone de evidencias cinéticas que indican la relación del inhibidor con respecto a los sitios de unión de nucleótidos y del Pi.

Al respecto, Krull y Schuster utilizando F_1 soluble encontraron que otros inhibidores de la ATPasa como el AMP-PNP, Cr ATP y Cr ADP, tienen un mismo sitio de unión que postulan como un sitio regulador diferente al sitio catalítico (114), parece ser que el inhibidor no se une a ninguno de estos sitios directamente, aunque sugieren que su acción podría ser cercana al sitio regulador. Todas estas posibilidades se desprenden del análisis cinético con pares de inhibidores. Por otra parte, Klein y cols. (115) han demostrado que en la F_1 en complejo con la proteína inhibidora, se inhibe el intercambio de nucleótidos. Ferguson y cols.(29) encontraron inhibido el recambio de nucleótidos endógenos fuertemente unidos en PSM de Mg ATP y aumentado en PSM expuestas a succinato.

Quizás estos datos sean consistentes con los del grupo de Slater (85) que proponen que dos moléculas de aurovertina se unen a las partículas A, las cuales tienen bajo contenido de proteína inhibidora (inferido por su alta actividad de hidrólisis de ATP).

Con el Pi, el otro de los sustratos de las reacciones catalizadas por la ATPasa, Klein y cols. (115) encontraron que cuando el inhibidor se encuentra formando complejos con la enzima, el Pi no se une, esto ha sido explicado por los autores como una inducción por el inhibidor de un cambio conformacional sobre la F_1, generando un nuevo estado en la enzima al que ya no tienen acceso ni los adenin nucleótidos ni el P_i.

Koslov y Skulachev (25) han postulado la existencia de un grupo carboxilo en el sitio activo de la F_1, mediante el empleo de un reactivo soluble afín a los grupos carboxilos de las proteínas, el CMCD marcado y que se une modificando un residuo de la enzima, induciendo una inhibición de la actividad de ATPasa; cuando la F_1 soluble en complejo con el inhibidor se expone a CMCD, el reactivo no inhibe la hidrólisis de ATP, hecho que es interpretado por los autores como un efecto del inhibidor sobre un sitio que no es el catalítico, pero que interactúa con él, descartando que el inhibidor actúe sobre el sitio catalítico en base a que la inhibición por el inhibidor es del tipo no-competitivo con el ATP (115) y porque el CMCD actúa sobre el sitio catalítico.

Los datos de los que disponemos sugieren que aunque la proteína inhibidora se fija a la subunidad β (29,117) a la que se le reconoce la posesión del sitio activo, no se le une en este lugar sino en uno diferente y específico desde el cual puede interactuar a través de cambios conformacionales o efectos alostéricos.

Se han descrito modelos que intentan describir el modo de acción de la proteína inhibidora (109), pero todos adolecen de ambigüedades o huecos que los hacen poco satisfactorios.

Una contribución importante al conocimiento del papel del inhibidor en la fisiología de la ATPasa, es la hecha por los grupos de Harris (118) y Gómez-

Puyou (119). Con sus trabajos, establecieron que el retardo en la velocidad inicial de fosforilación en partículas submitocondriales y cloroplastos se correlacionaba con el desplazamiento de la proteína inhibidora.

Anteriormente se le reconocía a la proteína inhibidora como un inhibidor de la hidrólisis (25,86,98) postulándosele de hecho como un inhibidor unidireccional de la ATPasa (120), controlando el flujo de energía desde el ATP hasta los sistemas de transporte de iones y de electrones. Asami y cols. sugirieron ésto en base a que habían encontrado que la proteína inhibidora bloqueaba las reacciones de transferencia de energía promovidas por ATP, tales como la reducción de NAD^+ ligada a succinato, la transhidrogenasa de nucleótidos de nicotínamida, el aumento de fluorescencia del ANS y la actividad de hidrólisis de ATP, pero no la fosforilación oxidativa (medida por polarógrafo). Sin embargo cuando Harris y Crofts (121) midieron la fotofosforilación en cloroplastos con una metodología más precisa, encontraron la aparición de una fase de retardo anterior al establecimiento del estado estacionario y propusieron que este retardo se debía al desplazamiento del inhibidor desencadenado por "una entrada de energía". La funcionalidad del inhibidor adquirió nuevo sentido. Un año mas tarde Harris y cols. y Gómez-Puyou y cols. encontraron una clara correlación entre la aparición y duración del tiempo de retardo y el contenido de inhibidor de diferentes preparaciones de PSM.

La relevancia de este proceso queda manifiesta en la Sec. A del Cap. III de esta tesis, en el que se describen las condiciones en las que en mitocondrias aisladas, el $\Delta\mu H^+$ puede manifestarse en la actividad enzimática de la ATPasa a través de su efecto sobre el desplazamiento del inhibidor, este mismo fenómeno es estudiado en presencia de otro proceso que requiere ener-

gía y que es el transporte de Ca^{2+}, el cual puede efectuarse sin que haya activación de la hidrólisis de ATP por desplazamiento del inhibidor.

Permanece como dato desconocido si el inhibidor bajo el efecto del potencial electroquímico es liberado, desplazado o cambiado conformacionalmente. Pedersen argumenta que no se tienen evidencias directas que prueben tal disociación (109). Consecuentemente, no se sabe del proceso inverso, si es que existe: el de reasociación de la proteína inhibidora a la enzima.

En este punto se hace pertinente mencionar que Hatefi estudiando el complejo V (109) ha observado que la fracción endógena de proteína inhibidora que no es removida durante el proceso de extracción del complejo, es capaz de manifestarse inhibiendo la hidrólisis de ATP; este autor ha interpretado dichos resultados proponiendo la existencia de un complejo F_1-I, en el cual la proteína inhibidora mantiene latente su actividad. Asimismo se ha encontrado que las partículas de Fdo. 3 reaccionan con el anticuerpo dirigido contra el inhibidor (122).

En nuestro laboratorio también hemos encontrado evidencias que señalan que el inhibidor puede hallarse presente en PSM sin estar inhibiendo la hidrólisis de ATP (123).

El hecho de que se haya determinado que el inhibidor guarda una estequiometría de 1 mol por mol de F_1 soluble, manteniendo una interacción con la subunidad β (117), no descarta la posibilidad de otro sitio topográfico o de otro estado conformacional para el inhibidor con respecto al complejo ATPasa, ya que como es sabido y se ha mencionado antes, la F_1 soluble presenta claras y varias propiedades diferentes a la enzima de localización membranal.

magnitud y demarcación de los cambios conformacionales? ¿Cuál es el mecanismo de síntesis y la función de los nucleótidos unidos? ¿Cuál es el mecanismo de transmisión de H^+ por la F_o? ¿Cuál es la función que in situ pueden llevar a cabo los cationes o aniones sobre los sitios respectivos?, etc.

En esta tesis se agrupan los trabajos referentes a los temas que se han abordado en la introducción precedente. Los objetivos concretos que trataron de resolverse en los artículos que a continuación se exponen, son los siguientes:

1) El MMIQ, un compuesto hidrofóbico, de carga positiva, ¿es capaz de interferir con el sitio I de la cadena respiratoria?

2) ¿Reside en la ATPasa mitocondrial el sitio de estimulación de K^+?

3) El agua deuterada y el metanol como herramientas en el estudio de las interacciones hidrofóbicas que intervienen en la F_1 soluble y particulada.

4) El papel de la proteína inhibidora sobre la ATPasa y sobre otros mecanismos del transporte mitocondrial.

CAPITULO II

EL MMIQ Y EL K^+ COMO EFECTORES DEL SITIO I

SECCION A. Effect of 1-(p-methozybenzyl)-6,7 methylene dioxyisoquinoline on mitochondrial respiration.

SECCION B. Action of K^+ on soluble and particulate mitochondrial F_1. Its relation to oxidative phosphorylation.

brain levels of [^{3}H]α-MeDA occurred at approximately the same time as the non-amphetamine-like behavior (greatly reduced response to external stimuli, decreased motor activity, and signs of fatigue 6–18 hr after MDA administration to mice[1, *]) supports the hypothesis that α-MeDA may be the mediator.

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Effect of 1-(*p*-methoxybenzyl)-6,7-methylenedioxyisoquinoline on mitochondrial respiration

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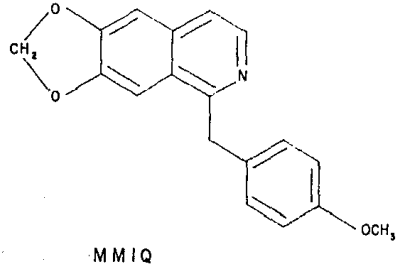
The isolation of an alkaloid extracted from a species of *Ocotea* (*lauracea*) which grows in the Amazon region, Brazil, has recently been reported. Its chemical structure is 1-(*p*-methoxybenzyl)-6,7-methylenedioxyisoquinoline (MMIQ) (Fig. 1)[1]. The compound inhibits the conversion of cyclic 3',5-AMP to 5'-AMP[1] and influences the contraction–relaxation cycle of smooth muscle. MMIQ is hydrophobic and possesses a positive charge at a pH around 7. Since certain hydrophobic compounds with a positive charge inhibit mitochondrial oxidative phosphorylation[2–4], the effect of MMIQ on the respiration and phosphorylation processes of mitochondria was explored. The results show that MMIQ inhibits the electron-transfer process of mitochondria.

Rat liver mitochondria were isolated in 0.25 M mannitol, 2 mM Hepes and 0.1% bovine serum albumin (pH 7.4). EDTA submitochondrial particles were prepared as described elsewhere[3]. Oxygen uptake was measured either with a Gilson or a YSI apparatus adapted with a Clark electrode. The ATPase activity of submitochondrial particles was measured in a mixture that contained 0.24 M mannitol, 10 mM Tris–HCl (pH 7.4), 11 mM ATP and 4 mM $MgCl_2$ in a final volume of 1.0 ml. The incubation time was 10 min at a temperature of 30°. The reaction was stopped with 6% trichloroacetic acid (final concentration). Inorganic phosphate was determined in the supernatant



MMIQ

Fig. 1. Chemical structure of MMIQ.

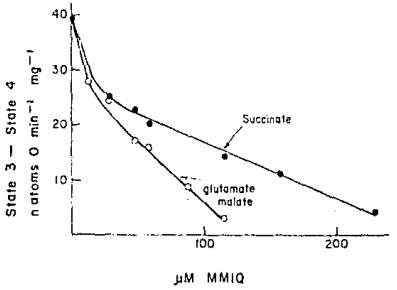


Fig. 2. Effect of MMIQ on the State 3–State 4 respiration of rat liver mitochondria. The incubation mixture contained, in a final volume of 2.0 ml, 10 mM glutamate, 10 mM malate, 5 mM phosphate (pH 7.4), 0.25 M mannitol, 1 mg of bovine serum albumin, 1.5 mg of mitochondrial protein and the indicated concentrations of MMIQ. State 3 was induced by the addition of 1 μmole ADP. Where indicated the mixture contained 10 mM succinate and 3 μg rotenone instead of glutamate-malate. Temperature was 27°. The results show enhancement of the respiratory rate attained by the addition of ADP to mitochondria incubated with the indicated concentration of MMIQ. For the effect of MMIQ on State 4, see text.

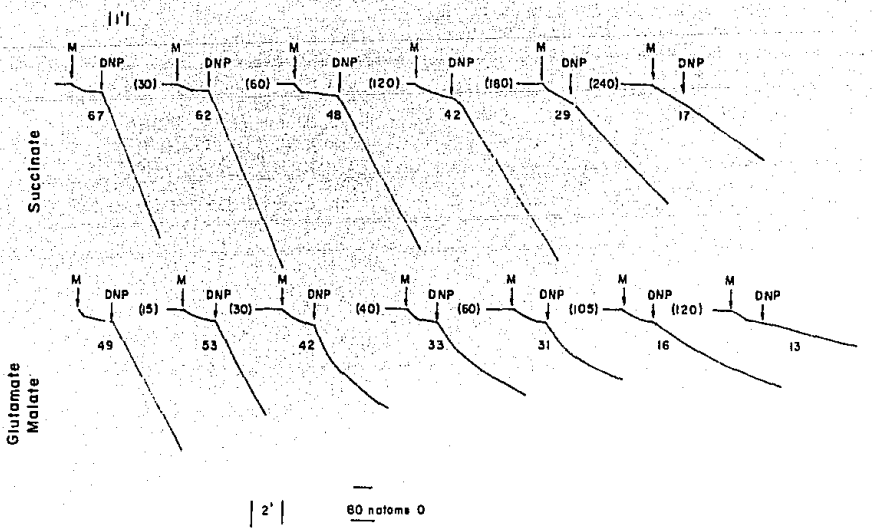


Fig. 3. Effect of MMIQ on uncoupler stimulated respiration. The incubation conditions were as in Fig. 2, except that 10^{-4} M DNP was added where shown. The numbers in parentheses indicate the μM concentration of MMIQ, while the numbers to the left of each trace show the rate of respiration (natoms O min^{-1} mg^{-1}) after the addition of DNP.

fraction according to Sumner [5]. MMIQ was added as an ethanolic solution; the amount of ethanol added did not modify the parameters studied (10 μl/ml of incubation mixture was the highest concentration of ethanol employed). At the highest concentration employed, MMIQ did not precipitate in the respective incubation mixtures. Each experiment was repeated at least three times with different mitochondrial preparations, and the results showed a variation of less than 10 per cent.

The experiments in which the effect of MMIQ on State 3 and State 4 respiration of mitochondria was assayed showed that State 3 respiration with glutamate-malate as substrate was strongly inhibited by MMIQ (Fig. 2), half-maximal inhibition being attained by approximately 30–40 μM MMIQ. State 4 respiration was not affected by MMIQ (data not shown).

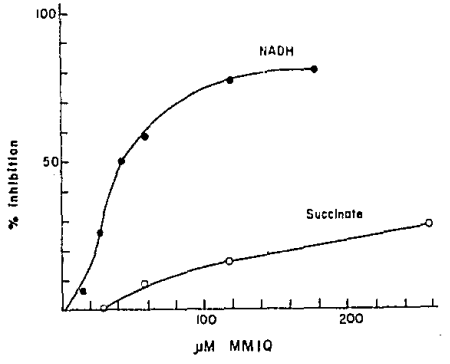


Fig. 4. Effect of MMIQ on the respiration of submitochondrial particles. The incubation mixture contained 0.25 M mannitol, 20 mM Tris–HCl (pH 7.4), and 1 μmole NADH or 10 mM succinate. The particles (0.8 mg protein) were incubated for 2 min with the indicated concentrations of MMIQ before the addition of substrate. The results express the per cent inhibition of respiration attained by the indicated concentrations of MMIQ. In the experiment shown, respiration in the absence of MMIQ was 354 and 280 natoms O min^{-1} mg^{-1} with NADH and succinate as substrate respectively.

With succinate (plus rotenone) as substrate (Fig. 2), State 3 respiration was also inhibited by MMIQ, but higher concentrations of the drug were required to induce an inhibition of State 3 respiratory rates equivalent to that attained with glutamate-malate as substrate. With succinate as substrate, concentrations of MMIQ above 100 μM increased State 4 respiratory rate from a rate in the control of 10 natoms O min^{-1} mg^{-1} to about 16 natoms O min^{-1} mg^{-1}.

The effect of MMIQ on respiration stimulated by 2,4-dinitrophenol (DNP) was examined (Fig. 3). With glutamate-malate as substrate, MMIQ also inhibited the DNP-stimulated oxygen uptake. However it should be noted that, within a certain concentration range of MMIQ (30–105 μM), DNP induces an enhancement of respiration that is not linear with time; that is, after an initial increase in the respiratory rate, the DNP-stimulated oxygen uptake starts to decline until it reaches the rates shown in Fig. 3. With succinate as substrate, MMIQ diminished the rate of the DNP-stimulated respiration, but at concentrations significantly higher than those that decreased the respiration with glutamate-malate.

The MMIQ-induced inhibition of electron transport in intact mitochondria could be due to inhibition of influx of substrates into the mitochondria or to an effect on the electron transport chain. Thus, its action on the respiration of submitochondrial particles in which permeability barriers do not exist was studied (Fig. 4). MMIQ inhibited by approximately 80 per cent the aerobic oxidation of NADH at concentrations which affected, although slightly, the aerobic oxidation of succinate.

MMIQ is an inhibitor of electron transfer in mitochondria. Its main action is on the NADH-CoQ segment of the respiratory chain. At higher concentrations it also affects the oxidation of succinate. It is of importance to point out that MMIQ does not affect the ATPase activity of submitochondrial particles (0.8 μmoles ATP hydrolyzed min^{-1} mg^{-1} under the conditions outlined above); thus, the

action of MMIQ would seem to be limited to the respiratory chain. However, if the data in Figs. 2 and 3 are compared to the results shown in Fig. 4, it may be observed that MMIQ inhibits succinate oxidation in mitochondria but not in submitochondrial particles. Therefore, the possibility that MMIQ affects the influx of succinate into the mitochondria cannot be fully discarded.

In comparison to other inhibitors of electron transport which preferentially act on the NADH-CoQ segment of the respiratory chain, MMIQ possesses some unique properties. It differs from rotenone and piericidin [6, 7] in that these two inhibitors are more potent than MMIQ [8], and in addition, MMIQ inhibits the oxidation of succinate by intact mitochondria. In this respect, it is more like octylguanidine [2, 3], which at low concentrations (30 μM) inhibits the coupled oxidation of NAD-dependent substrates and at much higher concentrations (300 μM) inhibits the coupled oxidation of succinate. Nevertheless, the inhibiting action of octylguanidine is reversed by uncouplers [9], while MMIQ inhibits uncoupler-stimulated respiration. Since MMIQ, octylguanidine and ethidium bromide possess a positive charge at a pH around 7, these three compounds most probably act on the same site or through a similar mechanism; however, it is probable that the binding of MMIQ to the membrane would be more stable owing to stronger hydrophobic interactions in addition to charge interactions. Thus, MMIQ would not be released from the membrane by the uncoupler-induced collapse of the electrochemical potential, as is the case for octylguanidine and ethidium bromide [2, 4].

Another important characteristic of MMIQ is that it inhibits electron transport at concentrations 50 per cent lower than those that inhibit phosphodiesterase activity [1]. This may indicate that MMIQ affects the contraction–relaxation cycle of muscle (see Ref. 1) by interfering with the mitochondrial energy-transducing system, particularly if it is considered that mitochondria, by regulating cytoplasmic Ca^{2+} levels, may affect muscle contraction [10]. This is a possibility that will be explored.

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Elevation of brain histamine levels by diaminopyrimidine inhibitors of histamine *N*-methyl transferase

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In the central nervous system (CNS) of mammalian species, histamine is metabolized primarily by methylation of the imidazole ring [1]. This reaction requires *S*-adenosyl methionine (SAM) as the methyl donor and is catalyzed by the enzyme histamine *N*-methyl transferase (HMT). In rats, however, tissues other than brain contain histaminase and thus have an alternative pathway for the metabolism of histamine. Among several antimalarial drugs that inhibit the methylation of histamine *in vitro*, pyrimethamine, a 2,4-diaminopyrimidine, has been reported to be a potent inhibitor of this enzyme [2]. The initial clinical studies of a pyrimethamine analog, metoprine, related to its evaluation as an anticancer agent, indicated that this compound produced CNS, cutaneous, and gastrointestinal toxicities [3–5], possibly involving inhibition of histamine catabolism. Similar CNS and cutaneous toxicities occurred during clinical studies of the anticancer agent triazinate, a diamino-*s*-triazine [6]. This report describes the effects of these anticancer agents on the activity of HMT *in vitro* and *in vivo*.

[^{14}C]methyl-SAM (sp. act. 57.8 mCi/m-mole) and [^{3}H]methyl-SAM (sp. act. 10.5 Ci/m-mole) were purchased from New England Nuclear; histamine, quinacrine and chloroquine were secured from Sigma Chemical Co., St. Louis, MO. Triazinate was obtained from the Drug Development Branch of the National Cancer Institute, Bethesda, MD. Pyrimethamine [2,4-diamino-5-(4′-chloro phenyl)-6-ethyl pyrimidine; BW 63U], metoprine [2,4-diamino-5-(3′,4′-dichlorophenyl)-6-methyl pyrimidine; DDMP; BW 197U], etoprine, the corresponding 6-ethyl analog of metoprine (DDEP; BW 276U) and triazinate [α-2-chloro 4-[4,6-diamino-2,2-dimethyl-*s*-triazine-1(2H)-yl]phenoxyl]-*N*,*N*-dimethyl-*m*-toluamide ethanesulfonic acid; TZT; NSC 139105] were dissolved in 0.01 N HCl prior to use.

HMT was partially purified from the cerebral cortex of bovine brain, using the ammonium sulfate fractionation and dialysis steps described by Brown *et al.* [7]. HMT incubation mixtures consisted of 10 μmoles of sodium phosphate buffer, pH 7.4, 15 nmoles histamine, 14.2 nmoles [^{14}C]-SAM, enzyme and, where appropriate, inhibitor, in a total volume of 300 μl. The reaction was initiated by the addition of [^{14}C]-SAM and incubated for 15 min at 37°. The reaction was terminated by the addition of 0.5

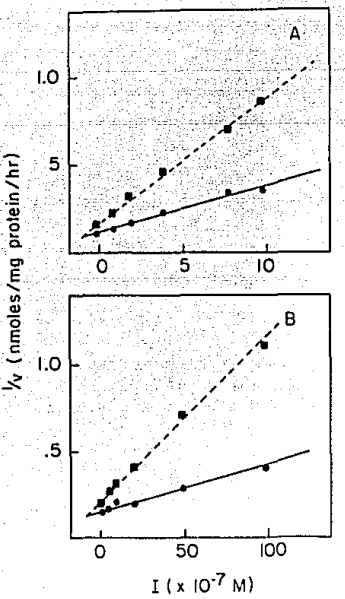

Fig. 1. Dixon plots of the effects of metoprine and triazinate on the activity of HMT. Values are the means of duplicates for each point. Panel A: metoprine (DDMP); panel B: triazinate. Key: ●——●, 5×10^{-5} M histamine; ■——■, 1×10^{-5} M histamine.

ml of 0.5 M borate buffer, pH 11, and the radioactive product was extracted with 6 ml toluene–isoamyl alcohol (1:1). Four ml of the organic extract was counted in 10 ml of an omnifluor–toluene scintillation mixture containing 2 ml of absolute ethanol. Kinetic studies were carried out at histamine concentrations ranging between 5×10^{-6} and 5×10^{-5} M, since higher concentrations of histamine have been shown to inhibit the enzyme [8]. The K_m for histamine was determined using the method of Lineweaver and Burk [9], and the various K_i values were determined by the method of Dixon [10].

To evaluate the activity *in vivo* of the diaminopyrimidines, male Sprague–Dawley rats (160–180 g) received a single oral dose of metoprine (10 mg/kg of body weight). The histamine levels in brain were determined using the enzymatic assay of Taylor and Snyder [11] as modified by Beaven *et al.* [12]. To avoid interference in the enzymatic assay, metoprine was removed from tissue extracts by chromatography on Dowex 50.

The effects of several drugs, all of which were found to be effective inhibitors of HMT isolated from bovine brain, are summarized in Table 1. Among these compounds, metoprine was the most potent inhibitor of the enzyme, producing greater than 90 per cent inhibition at a concentration of 10^{-5} M. In a series of 5-phenyl and 5-benzyl diaminopyrimidines, wide variations in potency as inhibitors of HMT were observed, and trimethoprim [13], an antibacterial agent, had relatively little activity as an inhibitor of HMT. For comparison, we include in Table 1 the effects of the antimalarial drugs chloroquine and quinacrine on the same enzyme preparation; these drugs were among the most effective inhibitors *in vitro* of HMT, as described previously [2].

The method of Dixon was used to determine the nature of the inhibition. The diaminopyrimidines and *s*-triazine were competitive inhibitors of the enzyme. This is illustrated graphically for metoprine and triazinate in Fig. 1. The kinetic data reveal that these agents are competitive with respect to histamine. Metoprine is the most potent inhibitor of the enzyme, having a K_i of 1×10^{-7} M. An important consideration concerning the likelihood of activity *in vivo* is that these compounds have K_i values at least 10-fold less than the K_m of histamine (Table 1). Studies are currently in progress to assess the effects of structurally related compounds on the metabolism of histamine and to identify the structural features necessary for these compounds to act as competitive inhibitors of HMT. In this regard, it should be noted that, in addition to diaminopyrimidines and triazines, several other classes of compounds act as inhibitors *in vitro* of HMT. We have observed that compounds containing the 4-aminoquinoline, aminoacridine, quinazoline or pyridopyrimidine nucleus are also potent inhibitors of the enzyme. Since a wide variety of compounds are capable of inhibiting this enzyme, one should be aware that unwanted side effects may be produced by some drugs via elevation *in vivo* of histamine levels.

Metoprine and etoprine are quite lipid soluble and readily enter the CNS. In rats, both drugs attained concentrations in the brain of approximately 2×10^{-5} M within 5 hr after oral administration of 10 mg/kg of body weight [14]. Studies on tissue distribution and elimination of metoprine in the rat have indicated a half-life of 17 hr in the brain [15]. In patients treated with metoprine for advanced malignancies, the plasma level was 10^{-5} to 10^{-6} M within a few hours after oral administration [5], and in some patients, headache and other CNS effects that may be histamine related persisted during the period of peak drug levels in the blood. Thus, these compounds are capable of reaching concentrations *in vivo* that result in significant inhibition of HMT activity *in vitro*.

The results illustrated in Fig. 2 show that metoprine, the most effective inhibitor *in vitro* of HMT, is capable of producing *in vivo* a marked elevation of brain histamine

Table 1. Inhibition of histamine methylation by several anticancer agents and antimalarial drugs*

	Per cent inhibition				K_i
	10^{-7} M	10^{-6} M	10^{-5} M	10^{-4} M	$\times 10^7$ M
Pyrimethamine	8	18	69	94	9.0
Metoprine	30	69	93	98	1.0
Etoprine	14	52	84	96	7.6
Triazinate	9	46	82	97	6.0
Quinacrine	0	25	79	97	
Chloroquine	0	9	55	89	

* For comparison, the K_m for histamine was 9.5×10^{-6} M under the same experimental conditions. The concentration of histamine used was 5×10^{-5} M.

ACTION OF K^+ ON SOLUBLE AND PARTICULATE MITOCHONDRIAL F_1 ITS RELATION TO OXIDATIVE PHOSPHORYLATION

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A number of investigators have shown that oxidative phosphorylation in mitochondria can be modulated by potassium ions (for a review, see Gomez-Puyou and Tuena de Gomez-Puyou, 1977). However, the mechanism by which this cation exerts its effect is not clear and indeed conflicting reports have appeared as to whether K^+ causes favorable or detrimental effects on oxidative phosphorylation (Pressman and Lardy, 1952; Blond and Whittam, 1964; Krall *et al.*, 1964; Papa *et al.*, 1969; Gomez-Puyou *et al.*, 1972; Aiking *et al.*, 1977). We have examined the role of K^+ in the oxidative phosphorylation of yeast mitochondria (*Saccharomyces cerevisiae* strain D311-3A); in these organelles the synthesis of ATP during coupled electron transport responds very dramatically to K^+ and hence the study of the action of K^+ is greatly facilitated. It has been found that K^+ affects oxidative phosphorylation by increasing the rate of electron transport in the NADH-CoQ span of the respiratory chain and also affects the ATPase complex, acting at the level of the F_1 component.

ACTION OF K^+ ON THE RESPIRATION AND PHOSPHORYLATION OF INTACT YEAST MITOCHONDRIA

With ethanol as the substrate, K^+ induces a marked enhancement of the respiratory rate, without affecting the ADP:O ratios (Table 1). With succinate as substrate, the effect of K^+ is different; it does not enhance the rate of respiration, but it does increase the ADP:O ratio.

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TABLE 1. EFFECT OF KCl ON OXIDATIVE PHOSPHORYLATION OF YEAST MITOCHONDRIA[a]

Addition	State 4	State 3[b]	ADP:O	Phosphorylation Rates
		Ethanol		
-	50	88	1.7	150
40mM KCl	65	121	1.6	183
		Succinate		
-	21	53	1.1	58
40mM KCl	22	46	1.6	74

[a]The reaction mixtures contained 600 mM mannitol, 10 mM phosphate (adjusted to pH 6.7 with Tris-base) and 10 mM substrate; where KCl was added, the concentration of mannitol was adjusted to maintain a constant tonicity. Mitochondria were prepared as described elsewhere (Pena *et al.*, 1977).

[b]State 3 rates were initiated by adding 1 μmole ADP.

Figure 1 shows the effect of various concentrations of K^+ on the State 3 and State 4 ratios of yeast mitochondria oxidizing succinate. Except at the higher concentrations of K^+ the rate of State 3 and State 4 respiration is not significantly affected, but the ADP:O ratio is significantly enhanced as the concentration of KCl is increased to 20mM.

According to current concepts of the mechanism of oxidative phosphorylation, it is believed that respiration is carried out by the various components of the electron transport chain, while the phosphorylation of ADP is mediated by the ATPase complex of the mitochondria. Accordingly, the action of K^+ on the electron transport chain and on the ATPase complex of the mitochondria was studied.

EFFECT OF KCl ON THE RESPIRATION OF SUBMITOCHONDRIAL PARTICLES

Table 2 illustrates the effect of KCl on the oxidation of NADH and succinate by submitochondrial particles. K^+ induces a more than three fold enhancement of the rate of respiration

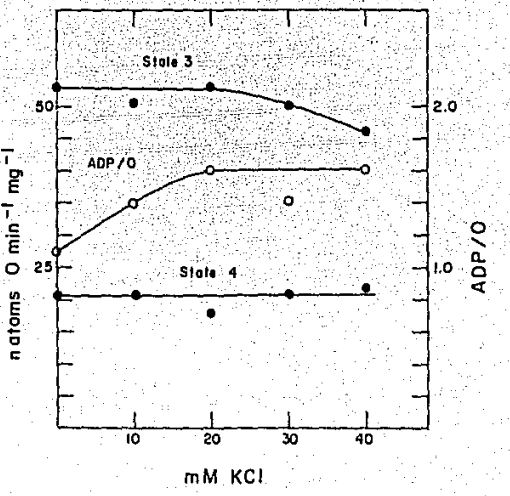

FIGURE 1. Effect of increasing concentrations of KCl on the oxidative phosphorylation of yeast mitochondria. The experimental conditions were described in Table 1.

with NADH as substrate, while the oxidation of succinate is little affected. This experiment was carried out under non-phosphorylating conditions with submitochondrial particles that had been obtained after sonication of mitochondria. These particles are devoid of its matrix enzymes and their membranes are inside-out; thus, the permeability barriers to substrates are largely non-existent.

From the data in Table 2, we may conclude that K^+ enhances the rate of electron transport, but since it does not affect the oxidation of succinate, it would appear that the site of action of K^+ is on the NADH to Coenzyme Q span of the respiratory chain and that it does not affect the chain on the oxygen side of cytochrome *b*.

In mitochondria from liver and heat a similar action of K^+ has been described (Lotina *et al.*, 1973), but our results suggest that in yeast mitochondria, which are rotenone-insensitive and show ADP:O ratios less than 2 with NAD-dependent substrates, neither the rotenone-sensitive site nor site 1 phosphorylation is involved. In any case an effect of K^+ on the NADH-CoQ span probably accounts for the stimulating action of

TABLE 2. EFFECT OF KCl ON THE RESPIRATION OF SUBMITOCHONDRIAL PARTICLES[a]

mM KCl	Respiration[b] natoms O min^{-1} mg^{-1}	
	NADH	Succinate
–	61	64
30	136	81
60	179	71
90	200	55

[a]The particles were prepared by sonication of mitochondria for 1 min in 250 mM sucrose, 1 mM EDTA (pH 8.0). The suspension was centrifuged for 10 min at 10,000 ×g and the supernatant solution was centrifuged for 105,000 ×g for 60 min. The final pellet was suspended in 0.25M sucrose.

[b]Respiration of the particles was assayed in media that contained 0.25M sucrose, 10 mM Tris-HCl (pH 7.3) and 1 μmole of NADH or 10 mM succinate.

the ion on the respiration of intact mitochondria oxidizing ethanol. On the other hand, this action of K^+ does not explain why, in the absence of modification of respiratory rates with succinate as the substrate, the phosphorylation of ADP in intact mitochondria is importantly enhanced by K^+. It was therefore necessary to explore whether potassium ions affect the functioning of the ATPase complex of the mitochondria.

EFFECT OF K^+ ON THE ATPase ACTIVITY OF MITOCHONDRIA

The ATPase activity of yeast mitochondria is enhanced by K^+ (Fig. 2). In many experiments and with mitochondria from several strains of yeast, we have observed 60 to 80% stimulation of ATPase by K^+. In intact mitochondria the activity both with and without K^+ are linear with time; concentrations of KCl of about 20 mM are required to induce half-maximal activation.

The concentrations of KCl that stimulate ATPase activity are in the range required for stimulation of oxidative phosphorylation in intact mitochondria. Accordingly, it was thought

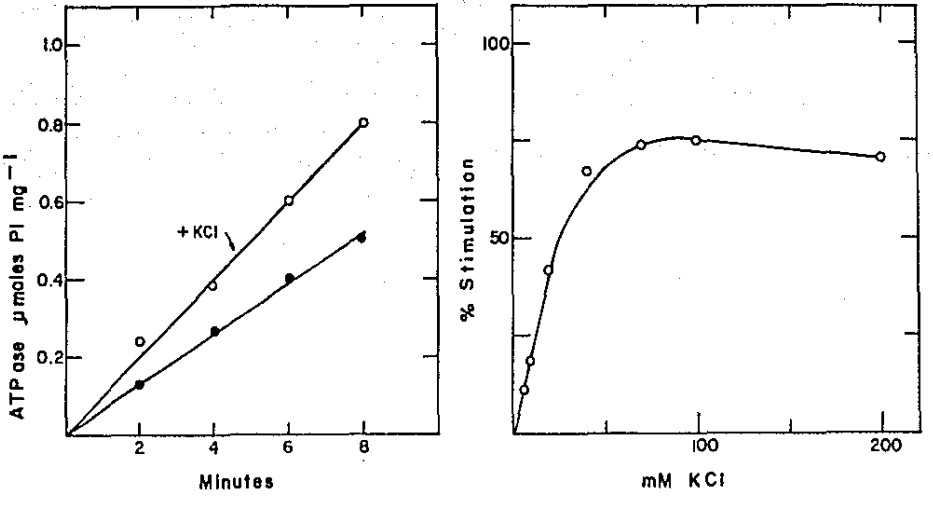

FIGURE 2. Effect of KCl on the ATPase activity of yeast mitochondria. Mitochondria were incubated in 250 mM mannitol, 10 mM Tris-HCl (pH 8.1), 5.5 mM ATP and 4 mM $MgCl_2$. On the left the mixture contained 70 mM KCl where indicated; on the right the incubation time was 4 min. The reaction was stopped with 6% trichloroacetic acid (final concentration) and inorganic phosphate determined according to Sumner (1944).

that these effects of KCl may be related and therefore the action of KCl on the ATPase complex of the mitochondria was examined. In recent years, elegant work has been carried out by several groups of investigators on the structure and functioning of the ATPase complex of many energy transducing membranes (for a review, see Pedersen, 1972). One of the most remarkable features of this enzymatic system is that the functioning of the complex is highly dependent on the subunit structure as well as on the arrangement of the various subunits that constitute the ATPase complex. This complex consists of two main structures. One is embedded within the membrane and requires very drastic procedures, such as treatment of mitochondria or submitochondrial particles with Triton X-100 in order to release it from the membrane. The other is a component that is soluble in water and is readily detached from the membrane by sonication.

According to the chemiosmotic concept of oxidative phosphorylation, the function of the membrane component (Mitchell, 1973; Mitchell, 1974) is to channel or transport protons from the outer part of the mitochondrial membrane to the catalytic site responsible for the synthesis of ATP, which resides in the soluble component (F_1). With respect to the action of K^+ on the ATPase complex of the mitochondria, it was of interest to determine whether K^+ acted on the membrane section or on the soluble F_1 component.

Table 3 shows the effects of oligomycin and K^+ on the ATPase activity of mitochondria which were subjected to sonication with ultrasonic irradiation for various periods. It was observed that after one minute of sonication, the sensitivity of the mitochondrial ATPase to oligomycin was almost abolished. This would be expected of a system in which F_1 had been released from the membrane, since its modulation by the membrane factor, as measured by sensitivity to oligomycin is lost.

The experiment also shows that despite the loss of sensitivity of the system to oligomycin, K^+ stimulated the ATPase activity. It may be concluded that K^+ stimulates ATPase activity by acting on F_1, the soluble component of the mitochondria.

Accordingly, F_1 released from mitochondria by sonication was partially purified as previously described (Tuena de Gomez-Puyou and Gomez-Puyou, 1977). The enzyme obtained hydrolyzed approximately 40 μmoles of ATP per mg of protein per min under optimal conditions and in sodium dodecyl sulfate gels (Tuena de Gomez-Puyou and Gomez-Puyou, 1977) showed the subunit composition characteristic of F_1.

TABLE 3. EFFECT OF TIME OF SONICATION ON THE ACTION OF K^+ ON MITOCHONDRIAL ATPase[a]

	− K^+		+ K^+	
Minutes of Sonication	−Oligomycin	+Oligomycin	−Oligomycin	+Oligomycin
–	1.3	0.3	2.0	0.4
0.5	2.9	1.8	3.6	3.4
1.0	2.6	2.1	3.5	3.2
3.0	0.5	0.5	0.5	0.5

[a]Mitochondria suspended in 0.6 M mannitol were sonicated for the indicated times and their ATPase activity measured as in Fig. 2 with 70 mM KCl in the mixture and/or 20 μg oligomycin per mg of protein.

Other monovalent cations including Li^+, Na^+, K^+ and Rb^+, showed similar effects on the ATPase activity of partially purified F_1 from yeast mitochondria. In contrast, the activity of F_1 from heart mitochondria (Tuena de Gomez-Puyou and Gomez-Puyou, 1976) was stimulated only by cations with a definite atomic radius; K^+ and Rb^+ were the most effective. Thus, a clear difference in the specificity of the response to monovalent cations is observed between the yeast and the heart enzyme, although the two enzymes have similar subunit composition. Despite these differences the results confirm that K^+ acts at the level of the F_1 component (Fig. 3).

ATTEMPTS TO DETERMINE THE MECHANISM OF THE K^+ EFFECT AND ITS RELEVANCE TO OXIDATIVE PHOSPHORYLATION

These observations left unanswered questions regarding 1) the mechanism whereby K^+ activates the ATPase activity of F_1 and 2) the significance of the activation in the formation of ATP during coupled electron transport. The following experiments were designed to provide answers to these questions.

The ATPase activity of F_1 is known to be inhibited by ADP (Pullman *et al.*, 1960; Hammes and Hilborn, 1971), and we have confirmed this observation (Fig. 4). Inhibition by ADP is also

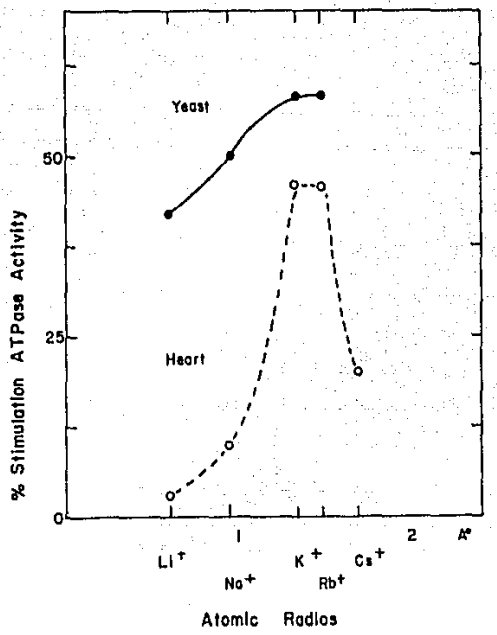

FIGURE 3. Effect of various monovalent cations on the ATPase activity of partially purified F_1. The reaction mixtures contained 5.5 mM ATP, 4 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.1), and 70 mM of the indicated chloride salts. The results with F_1 from heart mitochondria are included for comparison (for other details of the heart preparation, see reference Tuena de Gomez-Puyou and Gomez-Puyou, 1976).

observed in the presence of K^+ and the Lineweaver-Burk plots of percent inhibition against the concentration of ADP in the presence and absence of K^+ are very similar. The same Ki value for ADP is obtained in the presence and absence of K^+. Although these findings suggest that K^+ does not alter the affinity of the enzyme for ADP, a comparison of the *absolute* values indicates that the inhibition of ATPase activity by ADP is more significant in the presence of K^+ than in its absence. This may have some relevance to oxidative phosphorylation.

Potassium ions have a more profound effect on the response to changes in concentration of Mg-ATP. In the absence of potassium ions, a biphasic Lineweaver-Burk plot is observed (Fig. 4B), in agreement with the findings of Takeshige and

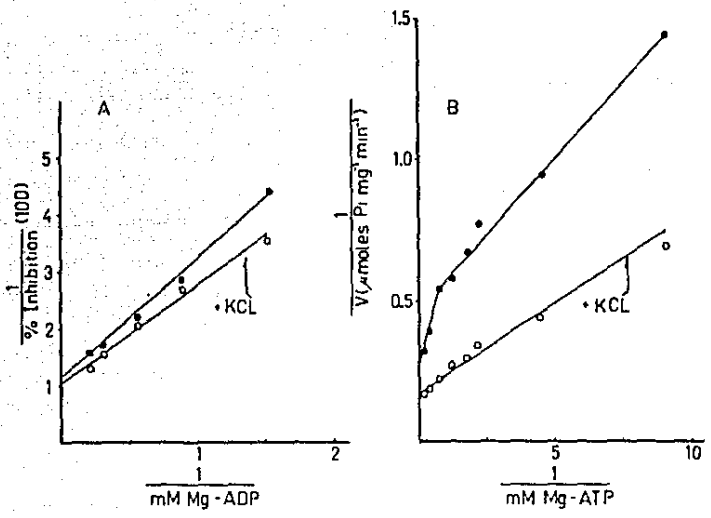

FIGURE 4. Effect of K^+ on the ATPase activity of F_1 at various concentrations of ADP and ATP. In A the incubating conditions were 10 mM Tris-HCl (pH 8.5), 4.2 mM Mg-ATP, and 8.4 mM $MgCl_2$ and the indicated concentrations of Mg-ADP and 70 mM KCl where shown. The percent inhibition by ADP is plotted taking as 100% the activity in the absence of ADP. In B, 10 mM Tris-HCl (pH 8.5), 70 mM KCl (where shown), and Mg-ATP (Mg-ATP/free Mg^{2+} = 1).

co-workers (Takeshige *et al.*, 1976). The data suggest that the enzyme may exist in two different conformations which are controlled by the concentration of ATP. In the presence of K^+, the biphasic pattern is almost completely eliminated. Thus K^+ appears to alter the response of a putative regulatory site, although explanations may be offered.

It is tempting to propose that a K^+-induced modification of the catalytical behavior of F_1 results in an increase of the phosphorylating rate of whole mitochondria. Evidence in favor of this possibility is afforded by the experiments shown in the Fig. 5, which may also explain why the effect of K^+ is less evident with mammalian mitochondria, than with yeast mitochondria. It has been reported that the phosphorylation that accompanies the oxidation of succinate by intact liver mitochondria is inhibited by octylguanidine (Papa *et al.*, 1975). Subsequent work (Tuena de Gomez-Puyou *et al.*, 1976) showed that octylguanidine inhibited ADP phosphorylation by interfering with the normal functioning of the ATPase complex and that the action of octylguanidine was exerted at the level of F_1.

Panel A of Fig. 5 shows that increasing concentrations of octylguanidine progressively inhibit the ATPase activity of liver mitochondria. The effect of octylguanidine on ADP phosphorylation shows markedly different kinetics and at low concentrations of octylguanidine (below 150 μM) the phosphorylation rate is not affected or slightly enhanced. The data suggest that in liver mitochondria the activity of the ATPase complex is *not rate-limiting*. The observations with octylguanidine are consistent with the results obtained with oligomycin, *i.e.* the curves for inhibition of coupled respiration and ATPase activity by increasing concentrations of oligomycin are sigmoidal and hyperbolic, respectively. The intrinsic ATPase activity in mammalian mitochondria appears sufficient to maintain maximal rates of phosphorylation.

In intact yeast mitochondria, increasing concentrations of K^+ increase the rate of phosphorylation and also cause an enhancement of ATPase activity. Thus in the absence of K^+, the activity of the ATPase complex may be rate limiting. The function of K^+ in yeast mitochondria would thus be to augment the rate of F_1 so as to induce maximal rates of phosphorylation.

In the strain employed for these studies the mitochondria are very resistant to oligomycin. This may be taken as evidence for a phosphorylating system that is not tightly coupled, and one way to compensate for the lack of efficiency would be to increase the overall rate of the process. In the light of the data reported here, this may be the role of potassium ions.

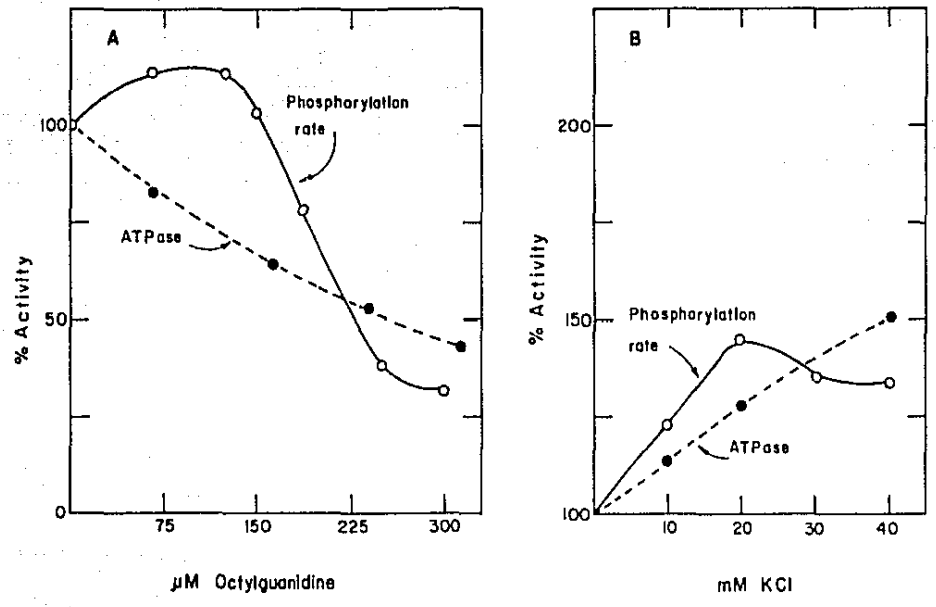

FIGURE 5. Effect of octylguanidine and KCl on the phosphorylation and ATPase activities. The experiments in A were carried out with liver mitochondria (for further details, see Papa et al., 1975); in B yeast mitochondria were employed and the experimental conditions were as described in Figs. 1 and 2.

In summary, we believe that the action of K^+ is to increase the catalytical activity of F_1 in the direction of ATP synthesis. However, K^+ in F_1 may also play a structural role. Thus K^+ not only increases the rate of phosphorylation but also the ADP:O ratios. In the presence of K^+, F_1 may adopt a conformation that inhibits the leakage of H^+ across the mitochondrial membrane.

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CAPITULO III

LAS INTERACCIONES HIDROFOBICAS Y LOS CAMBIOS CONFORMACIONALES EN LA ATPasa MITOCONDRIAL

SECCION A. Role of solvent (D_2O) on the kinetic and structural properties of mitochondrial ATPase

SECCION B. Conformational changes of soluble mitochondrial ATPase as controlled by hydrophobic interactions within the enzyme.

ROLE OF SOLVENT·(D_2O) ON THE KINETIC AND STRUCTURAL PROPERTIES OF MITOCHONDRIAL ATPase

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One of the questions that is often raised by students of membrane enzymology is why the properties of some enzymes isolated from a membrane differ from those of the membrane-bound enzymes. In this respect it is possible that the altered behavior of the enzyme is due to the separation of the protein from other membrane components, but also it is conceivable that the environment or solvent surrounding the membrane-bound enzyme induces some of its particular properties.

In order to explore the latter possibility, the effect of changes in the solvent on the soluble, oligomycin insensitive ATPase of mitochondria (F_1) was examined. This enzyme was chosen because it provides an excellent example of an enzyme whose properties are strikingly different in the particulate and soluble forms. For example, significant differences in the Km values for Mg-ATP, specificity towards various nucleosides triphosphates (Pedersen, 1976a), and energies of activation (Takeshige *et al.*, 1976) of the soluble and particulate F_1 species have been reported. Furthermore, in addition to the differences in sensitivity of the particulate and soluble enzyme to several inhibitors (Pedersen, 1975), the particulate

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enzyme is markedly resistant to low temperatures, whereas soluble F_1 is extremely sensitive to cold (Pullman *et al.*, 1960). Thus mitochondrial F_1 possesses some well-defined characteristics that indicate that the milieu in which the protein exists may affect the kinetic properties and the structure of the enzyme.

Micrographs of mitochondria obtained using the negative staining technique (Stoeckenius, 1970) show F_1 as a "knob" of approximately 90 $\overset{\circ}{A}$ that protrudes from the inner surface of the internal mitochondrial membrane. It has not been established with certainty, however, that this is the true state of the enzyme in the mitochondria, and the possibility that this picture results from the methodology employed has not been excluded. If, by changing the solvent used to study the soluble F_1, some or all of the properties of the membrane enzyme was to be reproduced, insight would be gained not only on the location of the enzyme in the membrane, but also on how this relates to ATP formation.

The study of the role of the solvent on the behavior of an enzyme presents some difficulties, mainly because the exposure of an enzyme to media of low dielectric constant often induces drastic and sometimes irreversible changes in the enzyme. Problems also exist with respect to the solubility of substrates and products in such non-polar media. In view of these problems,, we decided to study the behavior and characteristics of soluble F_1 in heavy water, D_2O, because the D-bonding of liquid heavy water is approximately 0.24 kcals per mole stronger than that of the H-bonding in ordinary water in the liquid phase. As a consequence of this higher strength of the D-bonding, heavy water is much more structured than H_2O. This conclusion is based in part on the differences that have been observed in the energies of hindered molecular rotation of H_2O and D_2O, and also on some of their bulk properties, such as melting point, viscosity and dielectric relaxation (Lewin, 1974).

It may therefore be expected that the solution of an enzyme in heavy water might induce two main alterations in the enzyme: the first, interactions of the polar groups of the enzyme with the solvent through D-bonding or H-bonding would be stronger in D_2O than in H_2O; this would also apply to those hydrogens in the protein that are substituted by deuterium in the short incubation times and are also capable of undergoing H-bonding. The second alteration to be expected also results from the higher structural order of D_2O. It is accepted by many workers (Tanford, 1973) that hydrophobic bonding originates mainly from the strong attractive forces between water molecules and that

the attraction *per se* of nonpolar groups plays only a minor role in the stability of a hydrophobic bonds. Thus if D_2O is more structured than H_2O, it would be expected that the hydrophobic bonding within a protein dissolved in D_2O would be more stable than in the same enzyme dissolved in H_2O.

Some evidence for this effect of D_2O on hydrophobic bonding has already been reported, Scheraga and his coworkers (Krescheck *et al.*, 1965) showed that the solubility of hydrocarbons is higher in D_2O than in H_2O, and also that the critical miscellar concentration of certain detergents is lower in D_2O than in H_2O.

Based on these properties of heavy water, two main factors should be considered when the effect of D_2O on an enzymatic system is studied: first, the higher stability of D-bonding with respect to H-bonding between the enzyme and the solvent and perhaps also between some groups within the enzyme, and second, the higher strength of hydrophobic bonding that may be induced within the enzyme following substitution of H_2O by D_2O.

EFFECT OF D_2O ON THE HYDROLYTIC ACTIVITY OF F_1

The ATPase activity of soluble F_1 obtained from baker's yeast decreases as the concentration of D_2O in the reaction mixture is increased (Fig. 1). At nearly 100% D_2O the enzyme is reduced by about 50-60%. These results are in agreement with other data reported in the literature in that rather high concentrations of D_2O are required to produce significant effects and also in that only partial loss of activity is observed. The effect of D_2O is fully reversible; if the enzyme after exposure to D_2O is transferred to H_2O, full enzymatic activity is restored. Therefore D_2O does not produce irreversible alterations of enzyme structure.

In order to probe in some detail the effect of heavy water on F_1, some of the kinetic characteristics of the enzyme were studied. The hydrolytic activity of F_1 from baker's yeast at various concentrations of Mg-ATP shows non-linear kinetics in a Lineweaver-Burk plot (Takeshige *et al.*, 1976; Tuena de Gomez-Puyou *et al.*, this volume). Fig. 2 shows that D_2O does not affect the kinetic pattern of the enzyme. The Lineweaver-Burk plot of the enzyme incubated in D_2O still shows two slopes, and moreover the Km of the system for Mg-ATP is not significantly modified by D_2O. The most obvious effect of D_2O would seem to be on the Vmax of the reaction.

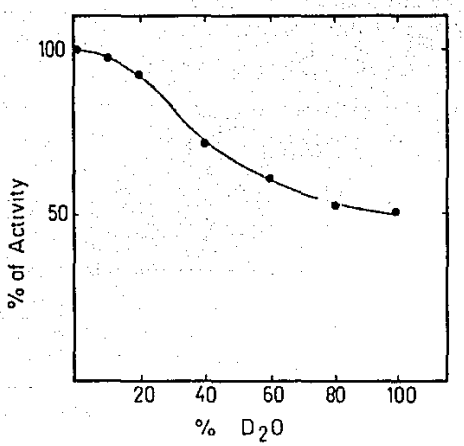

FIGURE 1. Effect of D_2O on the hydrolytic activity of soluble F_1. Soluble F_1 from baker's yeast was isolated and purified as described elsewhere (Reckterwald and Hess, 1977). Its activity was measured in 10 mM Tris-HCl (pH 7.7 or pD 8.1), 4 mM $MgCl_2$ and 5.5 mM ATP; incubation time 2 min., temperature 30°. Mixtures of H_2O and D_2O were prepared to yield the indicated content of D_2O. The reaction was stopped with trichloroacetic acid (6% final concentration) and inorganic phosphate was determined according to Sumner (1944). The + indicates the activity of F_1 incubated in D_2O for 10 min. and then measured in H_2O.

These observations raise the question as to the mechanism whereby D_2O reduces the enzymatic activity of F_1. There are several alternatives, and one of these would be the induction by D_2O of a structural rearrangement of the enzyme which would result in decreased enzymatic activity. Indeed, it has been shown that some modifiers of the catalytic properties of F_1, in particular anions, affect the functioning of F_1 by acting at a locus different from the catalytic site (Lambeth and Lardy, 1971; Pedersen, 1976b). The data show that the response of F_1 to an inhibitor or to an activator is not affected (Fig. 3). These findings, together with the observation that D_2O does not affect the Km of the enzyme for MgATP suggest that the enzyme has not suffered changes in structure that affect its general catalytic function.

As stated earlier, the possible effects of D_2O on the structure of an enzyme, include increased strength of D-bonding between the solvent and the protein as compared to H-bonding as

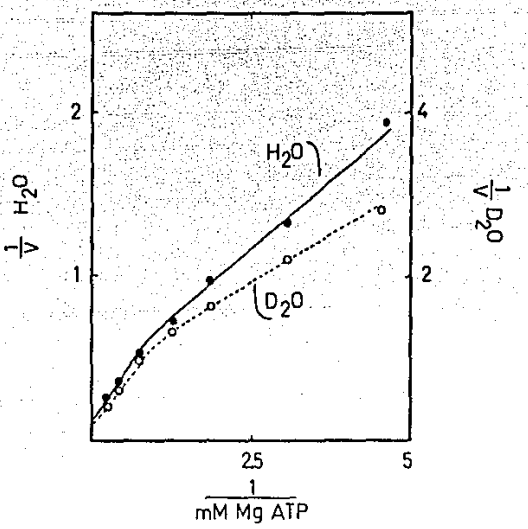

FIGURE 2. Effect of D_2O on the hydrolytic activity of F_1 at various concentrations of Mg-ATP. The reaction mixtures contained 10 mM Tris-HCl (pH 7.7 or pD 8.1), and the indicated concentrations of Mg-ATP, maintaining the ratio of Mg-ATP to free Mg^{2+} equal to 1. The temperature was 30° and the concentration of D_2O was 95%. Note that the scale for D_2O is 2 × that for H_2O.

well as the ability of D_2O to enhance hydrophobic bonding within the enzyme protein. We attempted to assess the contribution of these factors.

Oakenfull and Fenwick (1977) were able to calculate the changes in free energy of hydrophobic interactions at temperature between 278° and 320°K. They also calculated the free energy changes of electrostatic interactions at the same temperatures. It was found that as the temperature is increased, the value of ΔG for hydrophobic interactions decreases, that is, the strength of these interactions increases as the temperature of the system is raised. On the other hand, electrostatic interactions were weaker at the higher temperatures.

Accordingly, if heavy water decreases the enzymatic activity of F_1 through an enhancement of hydrophobic interactions, it would be expected that as the temperature of the system is

increased, the activity of the enzyme incubated in D_2O would remain at the lower level, regardless of increments in the temperature of the system. On the other hand, if the inhibition of enzymatic activity is a consequence of the increased strength of D-bonding relative to H-bonding, it would be expected that inhibition of enzymatic activity would be reversed by raising the temperature since the stability of H-bonding would be expected to decrease.

The results of experiments in which the effect of D_2O on the hydrolytic activity of soluble F_1 was measured at various temperatures are shown in Fig. 4. As expected, the hydrolytic activity of F_1 measured in H_2O increases as the temperature of the incubation system is increased up to about 45°C. At higher temperatures in H_2O, the enzyme activity declines. With the enzyme incubated in D_2O, a different picture is observed. At temperatures between 15° and 40°C, the activity of the enzyme in D_2O is lower than in H_2O, but above 40°C its activity increases very rapidly and indeed at about 55°C the activities in H_2O and D_2O are comparable. This behavior suggests that D_2O decreases the activity of the enzyme at the intermediate temperatures because of stronger interactions of the protein with the solvent through D-bonding. As the temperature is raised above 40°C, the strength of the D-bonding diminishes and the effect of D_2O diminishes.

The preceding experiments indicate that for full enzymatic activity the enzyme may require a certain degree of flexibility. When this flexibility is impaired through its interaction with the solvent, its activity declines. It should be emphasized that the overall catalytical properties of the enzyme are not altered by the solvent, only the rate at which it catalyzes the hydrolysis of ATP is diminished; other properties, including its sensitivity to cations and anions, and to the inhibiting effect of octylguanidine, as well as the Km values for Mg-ATP are not significantly modified (Figs. 2 and 3).

EFFECT OF D_2O ON THE COLD-SENSITIVITY OF SOLUBLE F_1

In addition to an impairment of the flexibility of the enzyme through its interaction with the solvent, another of the predicted effects of D_2O on a protein would be to increase the strength of intra-chain hydrophobic bonds. Thus it was considered of interest to explore whether this effect of D_2O could also affect the functional or structural properties of F_1, in particular its unusual cold-lability. The soluble enzyme is rapidly inactivated on exposure to temperatures of about 4°C.

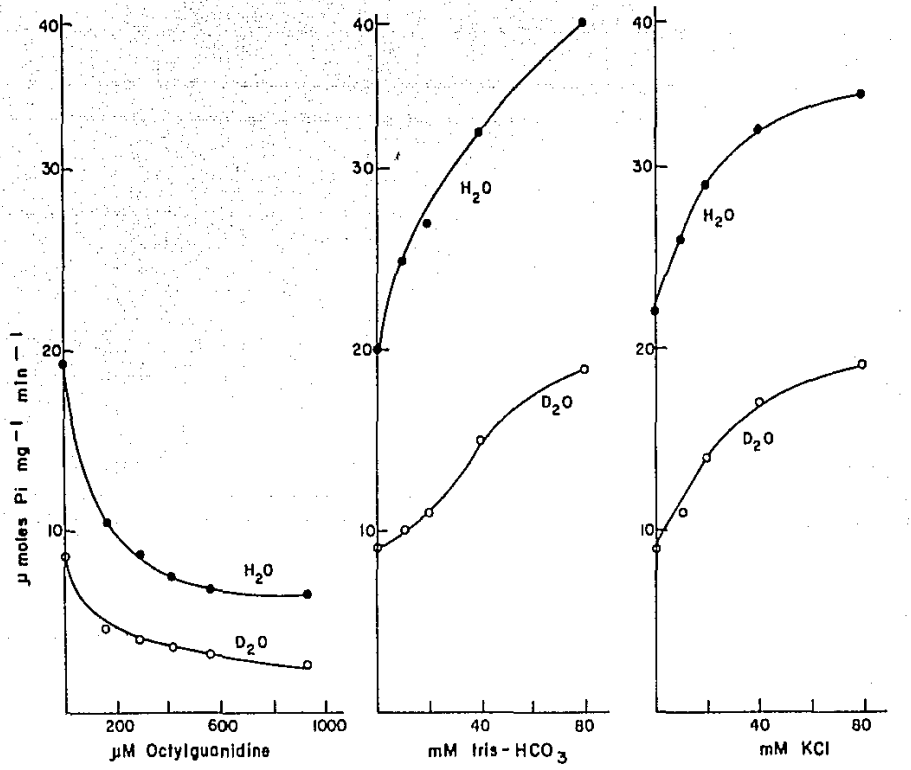

FIGURE 3. Effect of octylguanidine, HCO_3^-, and K^+ on the hydrolytic activity of soluble F_1 incubated in D_2O. The experimental conditions were as in Fig. 1 except that the mixtures contained 90% D_2O and the indicated concentrations of octylguanidine, tris-HCO_3 and KCl.

Penefsky and Warner (1965) showed that during this inactivation of soluble F_1 the enzyme dissociates into smaller molecular subunits. At low temperatures hydrophobic interactions are known to be weakened and it is likely that this contributes to the dissociation of F_1. The favorable effect of heavy water on the strength of hydrophobic interactions (Lewin, 1974) appeared to provide a useful tool to test this possibility. When the enzyme was incubated in H_2O or D_2O in the cold, it was found to lose activity rapidly in H_2O and most of the activity was lost in about 2 hours. In contrast, the activity of the enzyme incubated in D_2O was decreased by only 20%. Although Table 1 does not show it, even after 5 hours of exposure to low temperatures, the enzyme was 50% active.

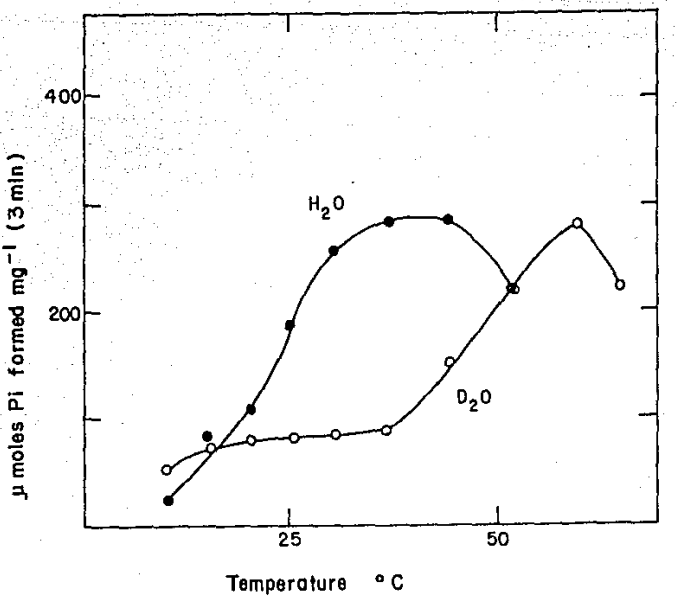

FIGURE 4. Effect of D_2O on the hydrolytic activity of F_1 at various temperatures. The experimental conditions were as in Fig. 1, except that the concentration of D_2O was 95% and the enzyme employed was derived from bovine heart. The incubation time was 3 min.

The protective effect of D_2O on the cold-induced inactivation of F_1 provides indirect evidence to support the suggestion that hydrophobic forces contribute to the interaction of the subunits of F_1. In order to confirm this possibility, we employed the fluorescent probe of ANS (8-amino naphtalene sulfonate). In media of low dielectric constant the fluorescence of ANS increases dramatically (Chapman and Dodd, 1971) and an increase in fluorescence of the dye in a given system is an indication that it has been transferred to a less polar environment.

If the subunit structure of F_1 is maintained by hydrophobic bonding between its subunits, it would be expected that once these subunits dissociate, some hydrophobic groups will appear. These newly exposed hydrophobic groups will be now accessible to ANS and the fluorescence of the system will increase. Table 1 shows not only that the fluorescence of ANS in the presence of the enzyme incubated in H_2O at 4° increases with time and

TABLE 1. EFFECT OF COLD ON THE ATPase ACTIVITY AND FLUORESCENCE OF SOLUBLE F_1 INCUBATED IN H_2O AND D_2O[a]

Time min	H_2O percent Activity	H_2O percent Fluorescence enhancement	D_2O percent Activity	D_2O percent Fluorescence enhancement
0	100	0	100	0
30	45	30	90	0
60	30	70	88	-
90	20	320	80	2
150	5	380	84	3

[a]Soluble F (from bovine heart) was incubated in an ice bath in 10 mM Tris-HCl (pH 7.3 or pD 7.7) and 100 mM KCl in H_2O or D_2O. At the indicated times aliquots were withdrawn and the ATPase activity measured in H_2O media as described in Fig. 1. For the fluorescence measurements the enzyme was incubated as above except that the mixture contained 10μM ANS (8-amino naphthalene sulfonate). At the indicated times, the fluorescence of ANS was recorded.

that the changes in fluorescence roughly parallel the inactivation of the enzyme. Thus, it is likely that the dissociation of the F_1-subunits results from the rupture of hydrophobic bonds that maintain the native structure of the enzyme. On the contrary, the enzyme incubated in D_2O, where loss of activity is small, the changes in ANS fluorescence are negligible. Since D_2O stabilizes hydrophobic interactions, the results are a further indication that hydrophobic bonding contributes to the stability of the subunit structure of F_1.

LOCATION OF F_1 IN THE MITOCHONDRIAL MEMBRANE

The results described in the preceding section suggest that the resistance to low temperatures of the membrane-bound form F_1 may be a consequence of stable hydrophobic bonding between its subunits which is somehow maintained by its situation in the membrane.

This raises the question as to whether the mitochondria F_1 is indeed on the outside of the membrane as has been concluded from micrographs obtained through the technique of negative staining show (Stoeckenius, 1970). We therefore carried out some electron microscope studies of mitochondria incubated in heavy water. When mitochondria were exposed to water treated with phosphotungstic acid dissolved in water by the usual procedures, F_1 is observed as a "knob" of approximately $90\overset{\circ}{A}$ protruding from the inner surface of the mitochondrial membrane (Fig. 5A).

If the same procedure is carried out in heavy water, these knobs are rarely seen and the membrane appears to be largely devoid of F_1 (Fig. 5B). Either F_1 was removed by treatment with D_2O, or in D_2O F_1 does not protrude from the inner surface of the mitochondrial membrane.

The removal of F_1 by D_2O does not appear to be likely since mitochondria and submitochondrial particles treated with D_2O show good rates of oligomycin sensitive ATPase (data not shown), which indicates that F_1 is present in the membrane. Additional evidence against the removal of F_1 by D_2O is provided by the micrographs shown in Fig. 5C. In 5B, mitochondria were incu-

FIGURE 5. Micrographs of mitochondrial membranes incubated in H_2O and D_2O. In A mitochondria were incubated in H_2O for 1 min. and fixed with 2% phosphotungstic acid dissolved in H_2O. In B and C mitochondria were incubated in D_2O for 1 and 3 min., respectively, and thereafter fixed with phosphotungstic acid dissolved in D_2O. The bar represents 0.1μM.

bated in heavy water for 1 min. and treated with phosphotungstic acid dissolved in D_2O. In 5C, the same mitochondria were also incubated with D_2O, but the exposure of the mitochondria to this osmotic shock was prolonged for 3 min. and thereafter treated with phosphotungstic acid dissolved in D_2O. In this latter condition a substantial number of "knobs" are clearly visible. In other words, mitochondria incubated for short periods of time show a "naked" membrane, but at longer incubation times, the 90°_{A} "knobs" become clearly visible.

The results thus indicate that F_1 is buried within the membrane and not protruding from the surface of the membrane. The

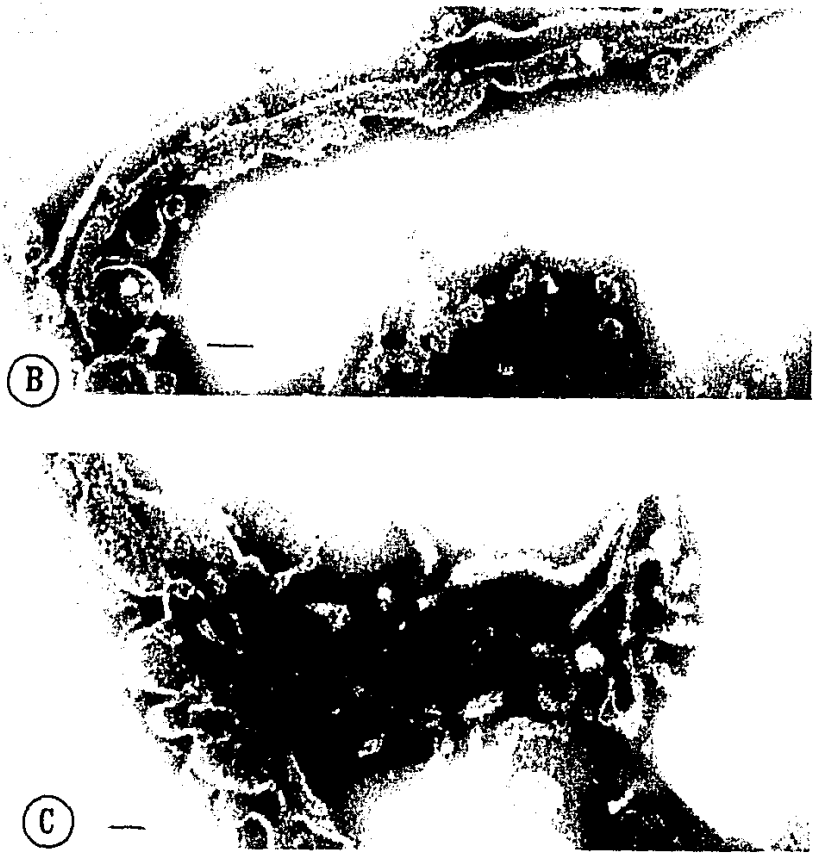

FIGURE 5B, C.

treatment of mitochondria with water and phosphotungstic acid (in H_2O) appears to induce a rapid extrusion of F_1 from the membrane, a process that is significantly delayed if all steps are carried out in D_2O. Heavy water may induce a stabilization of the membrane structures which permits their more accurate visualization.

The stabilizing effect of D_2O on membrane structure is not new. Hemolysis of erythrocytes has been reported to be delayed in heavy water. Also, the release of some constituent membrane enzymes by chaotropic salts requires higher concentrations of the salts in D_2O (Hanstein *et al.*, 1974). The stabilizing effect of D_2O on membrane structures, probably a consequence of the favorable effect of D_2O on hydrophobic interactions, may prove to be a useful tool in other electron microscope studies of membrane structure.

CONCLUSION

With respect to the functioning of F_1, the data reviewed here indicate that the activity of the enzyme is strongly affected by the nature of the solvent, possibly because a certain degree of flexibility is required for the enzyme to show its maximal activity. Recently, (Recktenwald and Hess, 1977) observed a lag period of about 100 msec before soluble F_1 exerted its catalytic activity. This may suggest that F_1 undergoes a transition from an inactive to an active state. In D_2O, this transition of F_1 from an inactive to an active state may be slower.

The results also indicate that the cold-lability of the soluble enzyme may be due to the sensitivity of hydrophobic bonding to low temperatures and that all or at least part of the subunit structure of F_1, required for full enzymatic activity, is maintained by such hydrophobic bonding.

Finally, with respect to the location of F_1 in the membrane, the electron microscope studies indicate that, under native conditions, the enzyme may be localized within the membrane and not, as previously supposed, protruding from the inner surface of the mitochondrial membrane.

More generally speaking, heavy water may have additional applications. For example, it may be employed in the isolation and storage of enzymes that are easily denatured. Because of its stabilizing action of membrane structure, it may be useful to study the natural structure of membranes by electron micro-

scopy or by other means, and it may also be applied to the study of other systems where stabilization of hydrophobic bonds is desired.

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CONFORMATIONAL CHANGES OF SOLUBLE MITOCHONDRIAL ATPase AS CONTROLLED BY HYDROPHOBIC INTERACTIONS WITHIN THE ENZYME

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SUMMARY

At 30° C soluble mitochondrial ATPase from baker's yeast shows non-linear kinetics with respect to Mg-ATP; the apparent Km values for Mg-ATP are 0.6 and 2.0 mM. At lower temperatures, 5°C and 12° C, the kinetics of the enzyme are linear with a Km for Mg-ATP of approximately 0.6 mM. Octylguanidine induces non-linear kinetics at 12° C. As octylguanidine and increases in temperature augment hydrophobic interactions within the enzyme, it is concluded that the strength of hydrophobic bonding within the protein regulates its conformational changes. Methanol activates the enzyme only at relatively high temperature which further indicates that the protein may exist in two active conformations.

INTRODUCTION

Pullman et al (1,2) were the first to isolate a soluble oligomycin insensitive ATPase (F_1) from the inner membrane of the mitochondria. The importance of this enzyme in oxidative phosphorylation became apparent when it was found that F_1 restored oxidative phosphorylation in mitochondrial membranes devoid of this factor (2). It is now established that F_1 possesses the catalytic site(s) for ATP formation and hydrolysis and thus, its mechanism of action is being extensively studied (for review see ref. 3). In this respect it has been proposed that during oxidative phosphorylation, the enzyme may undergo conformational changes (4,5) that are directly related to the formation of ATP, however at the present this possibility is a question of considerable controversy (3).

In this work it is shown that F_1 may exist in two forms, which may be distinguished by their distinctive kinetics toward Mg-ATP, and

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by their different sensitivity to methanol. In addition the results indicate that the change from one state to another is controlled by variations in the strength of the hydrophobic interactions that exist within the enzyme.

MATERIAL AND METHODS

Mitochondria from baker's yeast (La Azteca, S. A.) were obtained according to the procedure described by Tzagoloff (6) in 0.4 M sucrose, 50 mM tris- HCl (pH 8.2) and 1mM EDTA. The mitochondria were suspended in 0.25 M sucrose, 10 mM tris -HCl (pH 7.5) and 1 mM phenylmethylsulfonyl fluoride (PMSF) and sonicated for 1 min; the submitochondrial particles were isolated by centrifugation at 105,000 x g for 60 min. F_1 was solubilized by the method of Beechey et al (7), the water phase contained 0.15M sucrose, 2mM ATP, 2 mM EDTA and 1 mM PMSF (pH 7.3). Further purification was achieved by passage of the enzyme through a column of Sepharose-hexyl-ammonium (8), except that the elution medium contained 1 mM PMSF. The enzyme was stored as a 70% $(NH_4)SO_4$ precipitate. It was collected by centrifugation and the precipitate dissolved in 0.15M sucrose, 2mM ATP and 2mM EDTA (pH 7.4) and the ATPase activity measured in the conditions described under Results. After stopping the reaction with 5% trichloroacetic acid, inorganic phosphate was determined according to Sumner (9).

RESULTS

In agreement with Recktenwald and Hess (10) and Takeshige et al (11), the ATPase activity of yeast F_1 at various concentrations of Mg-ATP and at incubation temperature of 30° C shows a biphasic kinetic behavior in a Lineweaver-Burk plot (Fig. 1 A). The two Km values for Mg-ATP are approximately 0.6 mM and 2.0 mM. The kinetic pattern at 30° C has been consistently and repeatedly observed in twelve different preparations of soluble F_1 with almost identical results. However it has been found also that variations in temperature drastically alter the kinetic behavior of F_1. As shown in Figure 1A, at an incubation temperature of 12°C, and more clearly at 5°C, the Lineweaver-Burk plot is monophasic with a Km for Mg-ATP of approximately 0.6 mM, which apparently corresponds to one of the Km values of the enzyme incubated at 30°C.

It is known that the strength of hydrophobic bonding in a given system increases as the temperature is raised (12). Therefore one of the possible

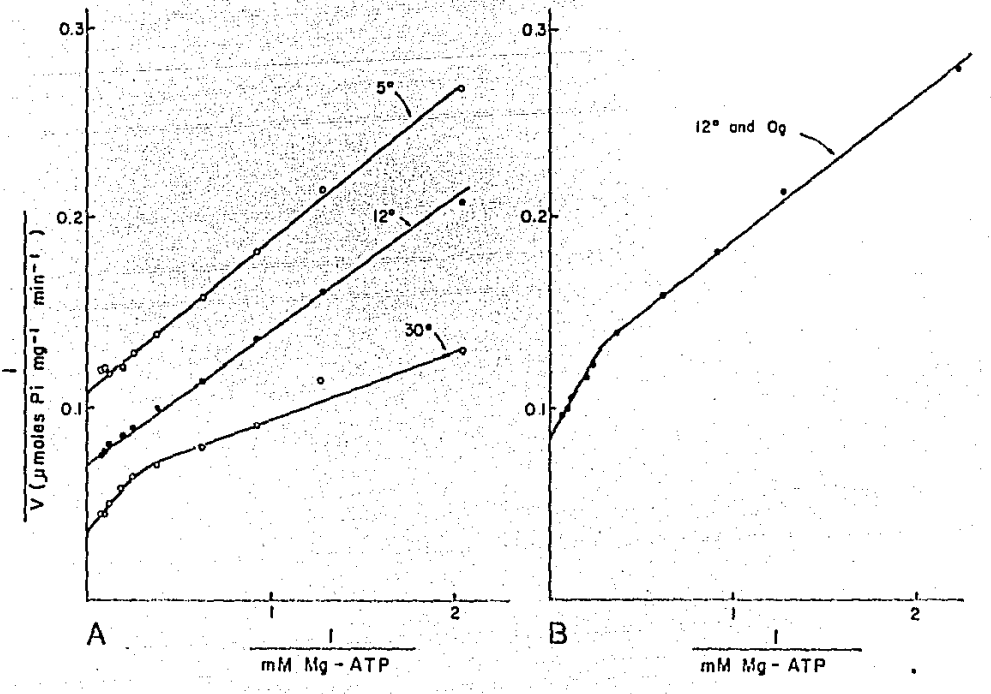

Figure 1. Lineweaver-Burk Plot for Mg-ATP of Soluble F_1. The experimental conditions were 10 mM tris-HCl (pH 8.5), the indicated concentrations of Mg-ATP shown maintaining Mg-ATP/free Mg^{2+} = 1. The incubation time was 1 min, at the indicated temperatures. The reaction was started by the addition of F_1 after temperature equilibration of the reaction mixture. The experiments at 30°C, 12°C, and 5°C were carried out with 15 μg 30 μg and 35 μg of enzyme respectively. In the experiment with octylguanidine (Og) 35μgm of enzyme were employed; the concentration of octylguanidine was 180 μM and the incubation temperature was 12°C

causes of the distinctive kinetic characteristics of F_1 at the temperatures assayed could be the different strength of hydrophobic interactions that exist within the enzyme (either in the interaction between its subunits or within one or another of its subunits). Indeed the sensitivity to low temperatures of soluble mitochondrial F_1 has been shown to be due to the weakening and rupture of the hydrophobic bonding that maintains the native structure of the enzyme (13). Accordingly it was thought that if the strength of hydrophobic bonding within F_1 controls the kinetics of the enzyme, any agent or condition that modifies the magnitude of hydrophobic interactions in the enzyme should also alter its catalytical properties.

Octylguanidine inhibits the hydrolytic activity of F_1 and protects the enzyme against the inactivating action of low temperatures, the latter effect of octylguanidine being due to an increase in hydrophobic interactions within the protein (14). Figure 1 B shows that at an incubation temperature of 12°C, octylguanidine inhibits ATPase activity, but also induces a change from linear to non-linear kinetics. Thus relatively high temperatures as well as octylguanidine at low temperatures induce non-linearity of enzyme kinetics. Apparently the common action of these two agents is to increase the strength of hydrophobic interactions in the enzyme.

The results of Figure 1 show that the enzyme may exist in two conformations i.e. a form characterized by a low Km for Mg-ATP (the low temperature form), and another that possesses a low and a high Km for Mg-ATP (the 30° C form). Therefore, it might be expected that these two forms could differ not only in their Km values toward Mg-ATP, but also in their response to agents that modulate the ATPase activity of F_1. Thus, the response to methanol of the enzyme incubated at various temperatures was explored, because methanol activates the enzyme through the removal of ADP, or via an alteration in the binding of ADP to F_1 (15), which suggests that the stimulation of the activity by methanol requires the presence of a binding site for ADP.

Table 1 shows that methanol fails to stimulate the ATPase activity of F_1 at temperatures below 20° C; at higher temperatures methanol induces a definite stimulation of ATPase activity. Apparently the appearance in the sensitivity of F_1 to methanol coincides with the change of monophasic to biphasic kinetics, which suggests that methanol affects only the form of the enzyme with the higher Km for Mg-ATP.

DISCUSSION

The results show that there are two forms of soluble F_1 one

Table I

Effect of Methanol on the ATPase Activity of Soluble F_1 at Various Temperatures

Temperature °C	μmoles Pi formed min^{-1} mg^{-1} - methanol	+ methanol	% of Activity in methanol
10	10.3	9.0	87
20	45.7	44.6	98
30	55.4	66.8	121
40	64.2	97.2	151
50	81.3	115.1	142

The experimental conditions were 5.5 mM ATP, 4 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.5), 15μg of F_1 and 15% methanol where shown. Incubation time was one min at the indicated temperature.

characterized by a low Km toward Mg-ATP and insensitivity to the activating action of methanol (the low temperature enzyme) and the other showing both low and high Km for Mg-ATP and the property of being activated by methanol. Although these findings can be explained solely on the basis of conformational changes, the results may also be consistent with the possibility that the enzyme in each of its two states possesses a different number of catalytic sites. The 30°C form would apparent possess two sites, while the low temperature form would possess only one site. The latter suggestion is reinforced by the observed lack of effect of methanol on the latter form of the enzyme. Since the activating action of methanol involves an alteration in the binding of ADP to F_1 (15), the absence of a stimulating action of methanol on the low temperature form would suggest that in this enzyme a binding site for adenine nucleotides is missing.

The data also indicate that F_1 undergoes conformational changes as a consequence of changes in the magnitude of hydrophobic bonding within the

enzyme. This is of interest because the solvent strikingly affects the kinetic and structural properties of F_1 (16). Therefore if during oxidative phosphorylation the enzyme undergoes changes in its location within the membrane (3), effects on the magnitude of hydrophobic bonding within the enzyme may induce reversible conformational changes accompanied by the appearance and disappearance of an adenine nucleotide binding site. Studies on the location of F_1 in the mitochondrial membrane will be presented elsewhere.

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CAPITULO IV

REGULACION DE LA ATPasa MITOCONDRIAL

SECCION A. Control of activity states of heart mitochondrial ATPase. Role of the proton-motive force and Ca^{2+}

SECCION B. Regulation of the ATP supported calcium uptake by heart and liver mitochondria.

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CONTROL OF ACTIVITY STATES OF HEART MITOCHONDRIAL ATPase

ROLE OF THE PROTON-MOTIVE FORCE AND Ca^{2+}

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Key words: F_1-ATPase; Mitochondrial ATPase; ATPase inhibitor; Ca^{2+} effect; Proton-motive force

Summary

The ATPase complex of submitochondrial particles exhibits activity transitions that are controlled by the natural ATPase inhibitor (Gómez-Puyou, A., Tuena de Gómez-Puyou, M. and Ernster, L. (1979) Biochim. Biophys. Acta 547, 252—257). The ATPase of intact heart mitochondria also shows reversible activity transitions; the activation reaction is induced by the establishment of electrochemical gradients, whilst the inactivation reaction is driven by collapse of the gradient. In addition it has been observed that the influx of Ca^{2+} into the mitochondria induces a rapid inactivation of the ATPase; this could be due to the transient collapse of the membrane potential in addition to a favorable effect of Ca^{2+}-ATP on the association of the ATPase inhibitor peptide to F_1-ATPase. This action of Ca^{2+} may explain why mitochondria utilize respiratory energy for the transport of Ca^{2+} in preference to phosphorylation. It is concluded that the mitochondrial ATPase inhibitor protein may exert a fundamental regulatory function in the utilization of electrochemical gradients.

Introduction

A protein that inhibits the hydrolytic activity of soluble and particulate mitochondrial ATPase was isolated by Pullman and Monroy [1]. It was sub-

Abbreviations: EDTA, ethylenediaminetetraacetic acid; F_1-ATPase, soluble, oligomycin-insensitive mitochondrial ATPase; FCCP, carbonyl cyanide 4-trifluoromethoxyphenylhydrazone.

sequently established that the protein inhibited all the ATP dependent reactions of mitochondria [2], and it was proposed that the inhibitor could act as a regulator of ATP synthesis and utilization [2,3].

More recently it was shown that the protein also inhibits the oxidative phosphorylation process of submitochondrial particles [4–6]. In chloroplasts, an almost identical action of the inhibitor has been observed on the photophosphorylation reaction [7]. In view of the possible role of the protein inhibitor on mitochondrial function, it is of importance to note that the experimental data indicated that the prior to the onset of phosphorylation, the ATPase exhibits a transition from an inactive to an active state. This transition was shown to be due to a proton-motive force induced dissociation of the inhibitor from the F_1 component of the mitochondrial ATPase [4,6].

In the light of these findings, several questions arise. One is as to whether the ATPase complex of intact mitochondria exists also in the inactive and active states, and, if so, whether the activity states of the ATPase could regulate the utilization of the proton-motive force for either ATP synthesis and/or some other energy-requiring process, such as ion transport.

In the latter respect, it should be recalled that, under certain conditions, mitochondria have been found to utilize energy derived from electron transport for driving Ca^{2+} influx, in preference to carrying out ADP phosphorylation [8,9].

The results of this work show that the ATPase complex of intact rat-heart mitochondria exists in inactive and active states, and some of the factors that control this transition are described. In addition the experimental findings provide a molecular explanation for the ability of mitochondria to accumulate Ca^{2+} in preference to oxidative phosphorylation.

Material and Methods

Mitochondria from the hearts of 4–6 rats were prepared either by the Nagarse method [9] (the authors are indebted to Dr. A. Vercesi for a practical demonstration of this procedure), or by grinding the minced heart with sand in 0.25 M sucrose, 1 mM EDTA pH 7.4. With respect to the ATPase experiments, both methods gave essentially the same results; however in mitochondria prepared by the Nagarse method, the respiratory control ratios were significantly higher (at least 4 in Nagarse prepared mitochondria and about 2 in the other preparation). Mitochondria were used within 1 h of their preparation.

ATPase activity was measured (unless otherwise stated) in 1.0 ml of an incubation mixture containing 0.8–1 mg mitochondrial protein, 0.125 M sucrose, 12.5 mM Tris-HCl (pH 7.4), and 3 mM ATP. The order of addition of the tested compounds, and other additions are described in the respective tables and figures. Trichloroacetic acid at a final concentration of 6% was added to stop the reaction and inorganic phosphate was determined in the supernatant according to Sumner [10].

Ca^{2+} uptake was determined by monitoring absorbance changes of 30 μM arsenazo (III) at 675–685 nm in a dual wavelength spectrophotometer [11].

Soluble mitochondrial F_1-ATPase was prepared as described elsewhere [12]; the preparation of the ATPase inhibitor protein has also been described [13].

Results

Activation and inactivation of the ATPase by electrochemical gradients

As shown in Table I, the ATPase activity of rat-heart mitochondria is increased several-fold by including the uncoupler FCCP in the incubation mixture. In the presence of glutamate-malate or succinate, the uncoupler stimulated ATP-ase activity becomes higher, whilst in the absence of the uncoupler a lower amount of inorganic phosphate is detected in the presence of the oxidizable substrates; the latter is most probably due to phosphorylation of ADP.

The activation of the uncoupler stimulated ATPase activity by glutamate-malate is inhibited by rotenone and antimycin, and that induced by succinate by antimycin. The stimulation effect of antimycin in the absence of FCCP may be due to an uncoupling effect of this antibiotic.

The aforementioned results indicate that electron transport induces activation of the ATPase complex. The results in Table II indicate that the proton-motive force derived from electron transport is the factor responsible for the activation, since FCCP effectively prevents the activating action of the substrates when added before electron transport is established. This conclusion is in agreement with the studies in Mg-ATP submitochondrial particles, in which it was shown that the ATPase could be activated by a proton-motive force via removal of the endogenous ATPase inhibitor from the F_1 component [4,5,14]. Also it agrees with previous findings of Bertina et al. [15] who used aurovertin as probe of conformational changes of particulate F_1 as induced by the energy state of the particles.

Fig. 1 shows that once the ATPase of intact mitochondria is activated, it remains in that state for a period of at least 7 min. These results indicate that the process is not reversed by collapse of the electrochemical potential, provided the enzyme is maintained in an active hydrolytic state. Moreover the experiment shows that the activity state of the ATPase may be evaluated by assay of

TABLE I

EFFECT OF OXIDIZABLE SUBSTRATES AND RESPIRATORY INHIBITORS OF THE ATPase ACTIVITY OF HEART MITOCHONDRIA

Mitochondria (0.72 mg) prepared by the Nagarse method were incubated in 1 ml of a mixture that contained 0.125 M sucrose, 12.5 mM Tirs-HCl (pH 7.4), and the indicated additions: rotenone 4 μg, antimycin A 0.5 μg, 5 mM glutamate/5 mM malate, or 5 mM succinate. After 3 min, 3 mM ATP ± 1 μM FCCP was added. Hydrolysis was allowed to proceed for 3 min.

Additions	ATPase (μmol P_i formed/mg)	
	—	+FCCP
None	0.09	0.54
Rotenone	0.13	0.35
Antimycin	0.25	0.50
Glutamate/malate	0.04	1.02
Glutamate + rotenone	0.16	0.60
Glutamate + antimycin	0.40	0.55
Succinate + rotenone	0.05	0.97
Succinate + antimycin	0.23	0.52

TABLE II

EFFECT OF THE ORDER OF ADDITION OF SUCCINATE AND FCCP ON THE ATPase ACTIVITY OF MITOCHONDRIA

Mitochondria (0.6 mg) prepared by grinding the tissue with sand were incubated in 1 ml of 0.125 M sucrose, 12.5 M sucrose, 12.5 M Tris-HCl (pH 7.4), 5 μg rotenone, and the indicated additions (A). After 3 min, 3 mM ATP plus the indicated additions in B were included in the mixture. Hydrolysis of ATP was allowed to proceed for 2 min.

Addition A	Addition B	ATPase (μmol P_i formed/mg/min)
5 mM succinate	ATP	0.04
1 μM FCCP	ATP	0.20
5 mM succinate	ATP/FCCP	0.38
1 μM FCCP/5 mM succinate	ATP	0.23

the uncoupler stimulated hydrolytic activity in relatively short times of incubation.

This property of the ATPase may be utilized to study its activity state as influenced by the metabolic condition of the mitochondria. In the experiment of Fig. 2 fresh mitochondria were incubated with glutamate-malate for 2 min, at this time rotenone was added, and this was followed by the sequential additions of succinate and antimycin. Samples of the suspension were withdrawn at pre-determined times and their uncoupler stimulated hydrolytic activity was explored.

The results show that the activity state of the ATPase oscillates in a fashion that is controlled by the build-up and collapse of an electrochemical gradient. Therefore electrochemical gradients would seem to be an important factor in the control of the number of ATPase molecules that exist in the active or

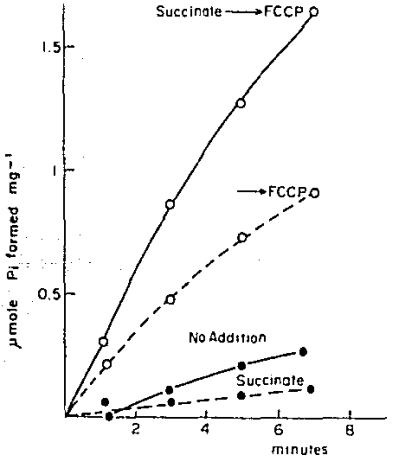

Fig. 1. ATPase activity of heart mitochondria preincubated with succinate. Mitochondria (3 mg) prepared by grinding the hearts with sand were incubated in 5 ml of 0.125 M sucrose, 12.5 mM Tris-HCl (pH 7.4), 4 μg rotenone, and 5 mM succinate as indicated. After 2 min, 3 mM ATP was added with an without 1 μM FCCP as shown by the arrow. At the times indicated aliquots of 1 ml were withdrawn and added to trichloroacetic acid; inorganic phosphate was assayed in the supernatant.

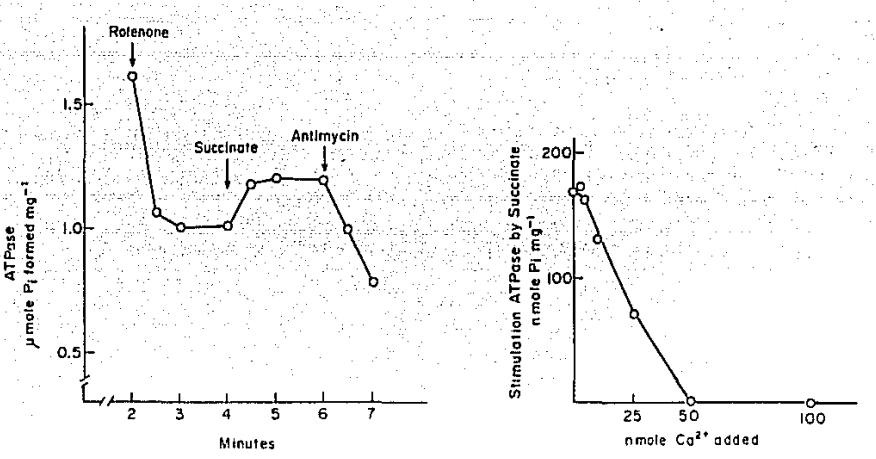

Fig. 2. Changes of ATPase activity of heart mitochondria as regulated by electron flow in the respiratory chain. Mitochondria (3.5 mg) prepared by the Nagarse method were added to 6 ml of incubation medium that contained 0.125 M sucrose, 12.5 mM Tris-HCl (pH 7.4), and 5 mM glutamate/5 mM malate. After two min and the times shown aliquots of 0.5 ml were withdrawn and added to 0.5 ml of 12.5 mM Tris-HCl pH 7.4, 1 μM FCCP and 3 mM ATP (final concentration). The hydrolysis of ATP was allowed to proceed for 3 min. At the arrows and immediately after withdrawl of the aliquot, 5 μg rotenone, 5 mM succinate, and 1 μg antimycin were added to the reaction mixture.

Fig. 3. Effect of Ca^{2+} on the activation of ATPase by succinate. Mitochondria (0.85 mg) prepared by grinding the hearts with sand were incubated in 1 ml of 1.25 M sucrose, 12.5 mM Tris-HCl (pH 8.4), 5 mM succinate, 2 μg rotenone, and the indicated concentrations of Ca^{2+}. After 3 min, 3 mM ATP and 1 μM FCCP were added; hydrolysis proceeded for 2 min. The stimulation of ATPase activity induced by succinate is plotted. Control tubes were incubated without succinate; the inclusion of Ca^{2+}, in the absence of succinate did not modify to a significant extent mitochondrial ATPase.

inactive state. Although the rate of inactivation and activation cannot be precisely determined by this methodology, the 'on' and 'off' rates seem to be compatible with those expected of a regulated process. Indeed in chloroplasts the activation rate of the ATPase via removal of the inhibitor has been shown to be in the millisecond range [7].

Effect of Ca^{2+} on the activity state of the ATPase

Mitochondria from a large variety of cells accumulate Ca^{2+} [16], and it is known that electrochemical gradients are the driving force for Ca^{2+} accumulatin [17]. As the activity state of mitochondrial ATPase seems to be controlled by the proton-motive force (Refs. 4, 5 and 14, and see also Fig. 2), it was considered of interest to explore whether Ca^{2+} transport affected the activity state of the enzyme.

For this purpose mitochondria were incubated with various concentrations of Ca^{2+} and with succinate. After 3 min ATP (+FCCP) was added and the hydrolysis of ATP was measured. Fig. 3 shows that Ca^{2+} effectively prevented the succinate induced activation of the ATPase. Parallel experiments carried out under identical conditions showed that in the presence of succinate, mitochondria could accumulate about 90 nmol of Ca^{2+}/mg in a period of about 1.5 min with 100 nmol Ca^{2+} added per mg of protein (data not shown).

The action of Ca^{2+} in inhibiting the succinate-induced activation could be

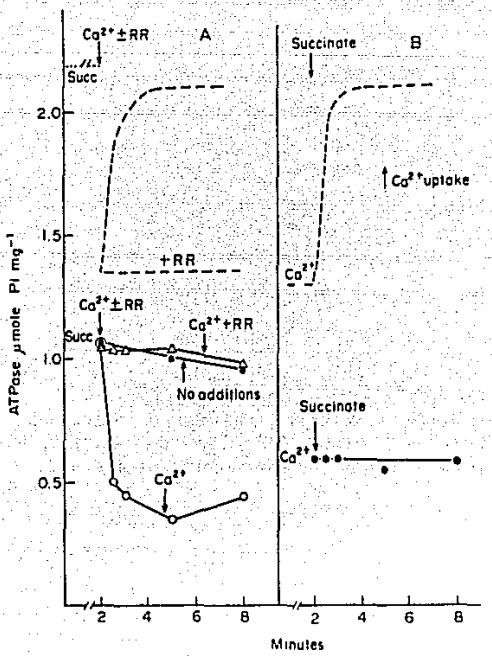

Fig. 4. Inactivation of mitochondrial ATPase by Ca^{2+} mitochondria (3.6 mg) prepared by the Nagarse method were incubated for 2 min in 3 ml of 0.125 M sucrose, 12.5 mM Tris-HCl (pH 7.4), 0.5 mM phosphate, 4 μg rotenone, and either 5 mM succinate (A) or 100 μM Ca^{2+} (B). At the indicated times 0.5 ml of this mixture was added to 0.5 ml Tris-HCl (pH 7.4), 3 mM ATP, and 1 μM FCCP (final concentration) and the hydrolysis measured in a 3-min period. After the first aliquot was withdrawn 100 μM Ca^{2+} was added (open circles in A); in the trace depicted with △———△ 100 μM Ca^{2+} plus 10 nmol ruthenium red (RR) was added, while in the trace illustrated with closed circles (A), no addition was made at 2 min. In B mitochondria were incubated with 100 μM Ca^{2+}, and 5 mM succinate was added at the arrow (2 min). The upper traces (dashed lines) indicate the absorbance changes at 675—685 nm of 60 μM arsenazo (III) of mitochondria incubated in conditions identical to those of the ATPase experiments. In A, Ca^{2+} uptake was started by adding Ca^{2+} (±ruthenium red (RR)) to mitochondria incubated for 2 min with succinate, while in B, uptake was started by adding succinate to mitochondria preincubated with Ca^{2+} for 2 min.

due either to internal Ca^{2+} causing e.g. an inhibition of the activity of adenine nucleotide translocase [18] or a prevention of the activation of the ATPase, due to the utilization of the electrochemical gradient for Ca^{2+} transport.

An action of internal Ca^{2+} on the translocase may be discarded, since the assay of the hydrolytic activity is carried out in the presence of FCCP which induces a release of Ca^{2+} from the mitochondria. Moreover the experiment of Fig. 3 shows that after the enzyme had been activated, the addition of FCCP (+ATP), which induces release of Ca^{2+}, does not reverse the activation. Therefore the results indicate that either internal Ca^{2+} or Ca^{2+} influx, or both may be responsible for inhibiting the activity of the ATPase complex.

To explore the latter alternatives, the experiment of Fig. 4 was carried out. Mitochondria were incubated for two min with succinate, at which time Ca^{2+} was added and the activity state of the ATPase was measured at various time intervals, by assay of the hydrolytic activity of aliquots withdrawn from the incubation mixture. Upon the addition of Ca^{2+} a sudden drop in the activity state of the enzyme was observed. The activity continued to be low for at least

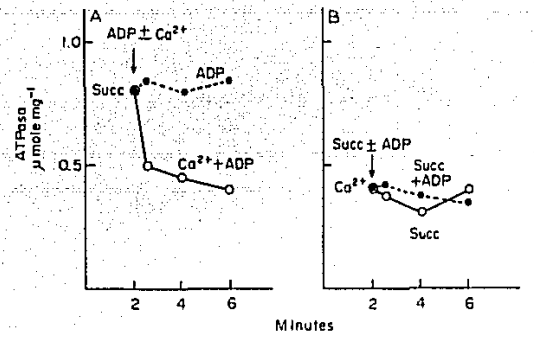

Fig. 5. Activity states of mitochondrial ATPase during state 4-state 3 transitions. Effect of Ca^{2+}. Mitochondria (3.8 mg) were incubated as in Fig. 4. The rest of the experimental conditions were also identical. In A, ADP (1 µmol) was added with and without 100 µM Ca^{2+} as shown to mitochondria preincubated for 2 min with succinate (Succ); whilst in B, 5 mM succinate ± 1 µmol ADP was added to mitochondria preincubated for 2 min with Ca^{2+}. Hydrolysis of the withdrawn samples was allowed to proceed for 2 min.

4 min; it should be mentioned that Ca^{2+} was completely taken up by the mitochondria in a period of about 60 s after its inclusion into the mixture (Fig. 4). The inhibition by Ca^{2+} was prevented by ruthenium red which inhibits the influx of Ca^{2+} into the mitochondria [19,20]. It was also observed that succinate did not activate the ATPase system of mitochondria previously incubated with Ca^{2+} (Fig. 4).

The results of Fig. 4 indicate that the influx of Ca^{2+} into mitochondria either prevents the activation of the ATPase or, alternatively, that it inhibits the enzyme by some other mechanism. In either case, this action of Ca^{2+} could account for the observed ability of mitochondria to accumulate Ca^{2+} in preference to oxidative phosphorylation [8,9].

According to the data of Fig. 5, it would seem that the activity state of the ATPase accounts for this property of mitochondria. Mitochondria incubated for 2 min with succinate show a high ATPase activity, this is not modified during a state 3-state 4 transition, as induced by a limiting amount of ADP (Fig. 5). On the other hand, the simultaneous addition of Ca^{2+} and ADP induces a strong and rapid fall of ATPase activity and the low activity persists for at least 4 min. In agreement with this observation, the findings of Fig. 5 indicate that mitochondria previously exposed to Ca^{2+} (and rotenone), in which the activity is low, the addition of succinate (with and without ADP), does not increase the activity of the ATPase complex (Fig. 5). In other experiments (not shown) it was found that the rate of ATP synthesis was diminished by about 60% after a completed Ca^{2+} cycle; this finding is in agreement with earlier observations by Vercesi et al. [9].

The preceding data indicate that Ca^{2+} influx may regulate the activity state of mitochondrial ATPase. However, the data show that the low activity, as observed in the presence of Ca^{2+}, persists even after Ca^{2+} has been taken up. Therefore it is conceivable that Ca^{2+} per se affects the activity of the ATPase, e.g. by preventing the release of the inhibitor from the enzyme.

The latter possibility was explored by measuring the effect of Ca-ATP on the interaction of soluble purified F_1-ATPase with the ATPase inhibitor. Horst-

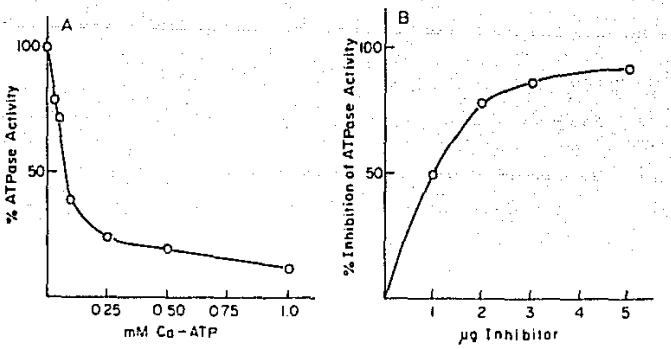

Fig. 6. Effect of Ca-ATP on the interaction of soluble F_1-ATPase with the natural ATPase inhibitor. In A, soluble F_1-ATPase (33 μg) was incubated with 4 μg inhibitor for 10 min in a mixture of 10 mM Tris-Mes (pH 6.5), and the indicated concentrations of Ca-ATP. Aliquots were withdrawn for measurements of ATPase activity. The specific activity of F_1-ATPase was 54 μmol/min/mg. In B, the same incubation conditions were employed except that 0.5 mM Ca-ATP was used, and the indicated concentration of the inhibitor was varied as indicated.

man and Racker [21] have reported that Ca^{2+} promotes the association of F_1 with inhibitor. The data of Fig. 6 show that the association depends on the concentration of Ca^{2+}-ATP added, and that at a concentration of 0.5 mM Ca-ATP the interaction becomes a function of the amount of inhibitor added. In this respect Ca^{2+} behaves similarly to Mg^{2+} (data not shown and Ref. 21).

Discussion

In this work, it is shown that the ATPase complex of intact heart mitochondria may exist in two states, which in reference to previous work [4] may be denoted as inactive and active. The present observations are in agreement with previous findings with particles [4], in which it was shown that the ATPase complex could undergo a reversible transition from the inactive to the active state through the reversible association of the ATPase inhibitor protein of Pullman and Monroy [1] with the F_1 component of the ATPase complex. As the presently described activity transitions of intact mitochondria are strikingly similar to those observed in particles [4], most likely the association and dissociation of the inhibitor from F_1-ATPase is responsible for the changes in activity of the enzyme in intact mitochondria.

The results of this work show that in intact mitochondria, it is possible to determine the activity state of the ATPase complex by measurements of its hydrolytic activity in the presence of an uncoupler. Using this method we observed that the activity state of the enzyme exhibits reversible changes. Although it has not been possible to determine the rates of activation or inactivation, the data suggest that these are compatible with a system that is of metabolic significance. In addition some of the factors or metabolic conditions that control the activity transition have been determined.

In agreement with previous observations in chloroplasts and submitochondrial particles [4—7], the present data indicate that the shift of the enzyme to the active state is mainly, if not solely, controlled by the establishment of electrochemical gradients. On the other hand, the inactivation of the system seems

to be more complex and subject to a larger number of variables. It has been observed that the collapse of the gradient favors the active to inactive transition, but in addition it has been found that the influx of Ca^{2+} also induces inactivation of the enzyme.

We have considered the possibility that the relatively small decrease in the electrochemical potential that occurs during Ca^{2+} influx in heart mitochondria [22] accounts for the inactivation of the ATPase, but alhough the drop in potential may account for the inactivation, other factors are certainly involved. If shifts in electrochemical gradients are responsible for the activity transitions, it would be expected that after Ca^{2+} uptake had taken place, the low activity of the ATPase would change to higher level. This was not the case; after Ca^{2+} had been accumulated the activity of ATPase remained at a low level. This observation suggested that Ca^{2+} may inhibit ATPase directly e.g. by preventing the dissociation of the inhibitor. Indeed we found, in agreement with Horstman and Racker [21], that Ca^{2+} effectively induces the association of the inhibitor with soluble F_1. Therefore it is conceivable that in mitochondria Ca^{2+} has a dual effect; its influx causes an association (or prevents the dissociation) of the inhibitor protein with F_1, due to a decrease in proton-motive force; and internal Ca^{2+} stabilizes, or induces the formation of the F_1-inhibitor complex. In this respect it is worth noting that Carafoli et al. (unpublished results) have observed that the ATP-supported Ca^{2+} uptake by heart and liver mitochondria may be regulated by the activity state of the ATPase.

The experiments described in this work also shed some light on the molecular events that account for the known feature of mitochondria to utilize their electrochemical potential for Ca^{2+} transport in preference to oxidative phosphorylation [8,9]. The inactivation of the ATPase by the inhibitory protein upon Ca^{2+} influx would explain why the proton-motive force is utilized for Ca^{2+} transport in preference to oxidative phosphorylation.

As the inhibitor protein has been isolated from mitochondria from a large variety of cells [1,2,23–27], it is possible that the protein exerts a general and important regulatory role in the overall functionality of mitochondria by controlling the pathways through which the proton-motive force is utilized.

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REGULATION OF THE ATP-SUPPORTED Ca^{2+} UPTAKE BY HEART AND LIVER MITOCHONDRIA

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SUMMARY

The ATP-supported Ca^{2+} uptake of heart and liver mitochondria preincubated in conditions in which electron transport had either been prevented by rotenone or antimycin, or induced by oxidizable substrates, has been studied. Mitochondria preincubated with respiratory inhibitors accumulate Ca^{2+} less efficiently than mitochondria preincubated with oxidizable substrates. The difference correlates with the degree of activation of the oligomycin-sensitive ATPase. The results indicate that the rate at which mitochondria take up Ca^{2+} in the ATP-supported system may be controlled by the reversible association of the inhibiting peptide (Pullman,, and Monroy, J. Biol. Chem., 238, 3762-3769) with the ATPase complex. Since this process appears to be modulated by the transmembrane electrochemical gradient, the latter may regulate the uptake of Ca^{2+} in a hitherto undescribed way.

INTRODUCTION

The transport of Ca^{2+} by mitochondria has been the subject of a large number of investigations (for a review see reference 1). It is supported by electrical gradients derived either from electron transport or from ATP hydrolysis; in the former case the process is oligomycin insensitive, in the latter it is completely prevented by the antibiotic. It is very likely that mitochondria play an important role in the regulation of Ca^{2+} levels in the extramitochondrial milieu (1). Therefore, knowledge of the factors that regulate the movements of Ca^{2+} across their membrane is of importance to determine the conditions by which mitochondria influence the Ca^{2+} concentrations of the cytosol.

Recently, it has been found that the mitochondrial ATPase may exist in two states: an "active" state in which the enzyme exhibits full hydrolytic and synthetic activities, and an "inactive" state, in which the two activities are totally depressed (2). The transition of one form of the enzyme to the other was found to be controlled by the so-called ATPase inhibitory peptide originally isolated by Pullman and Monroy (3). When the inhibitor peptide is associated to the ATPase, the enzymatic activities of the complex are completely abolished. Dissociation of the inhibitor from the ATPase, is induced by electrochemical gradients, and results in the complete restoration of the catalytic activities of the enzyme (2). In this work we will describe experiments indicating that under contitions leading to the "inactive" state of the ATPase, mitochondria show a low ability to accumulate Ca^{2+} in the ATP-supported system. By contrast, the rate of Ca^{2+} influx is significantly higher under conditions in which the ATPase is in an active state. The data suggest, a novel mechanism through which mitochondria may regulate the rate at which they take up Ca^{2+}. Apparently, this regulatory process is controlled by the shifting of the inhibitory peptide to and from the ATPase.

METHODS

Rat heart mitochondria were prepared by two methods. In the first, after disruption of the tissue by gentle grinding with sand in 0.25 M sucrose - 1 mM EGTA, 3 mM Hepes, pH 7.1, a standard differential centrifugation scheme was followed, with final resuspension of the mitochondria in 0.25 M sucrose, 3 mM Hepes, pH 7.1. The second method was that recently described by Vercesi et al. (4). The two methods yielded mitochondria that were nearly identical functionally. Rat liver mitochondria were prepared by homogenization of the minced liver in 0.25 M sucrose, 1 mM EDTA, 3 mM Hepes, pH 7.1. The mitochondria were washed twice and resuspended in 0.25 M sucrose, 3 mM Hepes, pH 7.1. $^{45}Ca^{2+}$ uptake was measured by Millipore filtration (the composition of the reaction media is detailed in the Results section). The filters were washed with cold 0.25 M sucrose, and countered in a scintillation counter. The general procedure for the measurement of the ATPase activity was the following. Mitochondria were incubated in mixtures of different composition (see Results section), and at the described times aliquots were withdrawn and added to a medium consisting of 3 mM ATP and 1 μM FCCP. After three minutes, the reaction was arrested with trichloracetic acid (6% final concentration) and inorganic phosphate was determined in the supernatant. The tetraphenylphosphonium-sensitive electrode used for the experiment of Figure 2 was prepared according to the indications given in (5).

RESULTS

ATP-Supported Ca^{2+} Uptake in Heart Mitochondria

Table I shows that the ability of heart mitochondria to ac-

TABLE I

Effect of Succinate and Antimycin A on the ATP-supported Ca^{2+} Uptake by Heart Mitochondria.

Preincubation Mixture	Addition at 5 min	Ca^{2+} Uptake (nmoles per mg protein)
---	Ca^{2+}/ATP	20.3
Antimycin	Ca^{2+}/ATP	10.2
Succinate/Antimycin	Ca^{2+}/ATP	13.3
Succinate	Ca^{2+}/ATP/Antimycin	24.8

Heart mitochondria (0.56 mg protein) prepared by the method of Vercesi et al. (4) were added to 1.0 ml of 0.25 M sucrose, 15 mM Tris-Cl pH 7.4, 4 µg rotenone and 5 mM succinate and/or 2 µg antimycin, as indicated. After five minutes of incubation at 25°C, 3 mM ATP, 100 nmoles $^{45}CaCl_2$, 2 µg antimycin were added. After one minute, an aliquot of the mixture was filtered through Millipore filters, washed, and the radioactivity determined in a scintillation counter.

In one minute, about twice as much Ca^{2+} is taken up by mitochondria that had been allowed to oxidize succinate during the 5 minutes preincubation, than by those that had been preincubated with antimycin A. The presence of succinate by itself is not the cause of the higher uptake of Ca^{2+}. Mitochondria incubated with succinate and antimycin, show a lower capacity to accumulate Ca^{2+} in the ATP-driven system, than those which had been preincubated with succinate alone, and in which Ca^{2+} uptake was initiated by the simultaneous addition of Ca^{2+}, ATP, and antimycin. Parallel experiments have shown that under these experimental conditions, antimycin induces a total and immediate arrest of the respiration.

The oxidation of glutamate-malate during the preincubation also induces a higher capacity to transport Ca^{2+} in the ATP-driven system (Fig. 1), as compared to mitochondria in which NADH oxidation was blocked by rotenone. In mitochondria preincubated with rotenone, the uptake of Ca^{2+} is higher than in those incubated with antimycin. This suggests that the oxidation of endogenous substrates may account for some of the ATP-supported Ca^{2+} uptake that is observed in the absence of added oxidizable substrates (see also Table I).

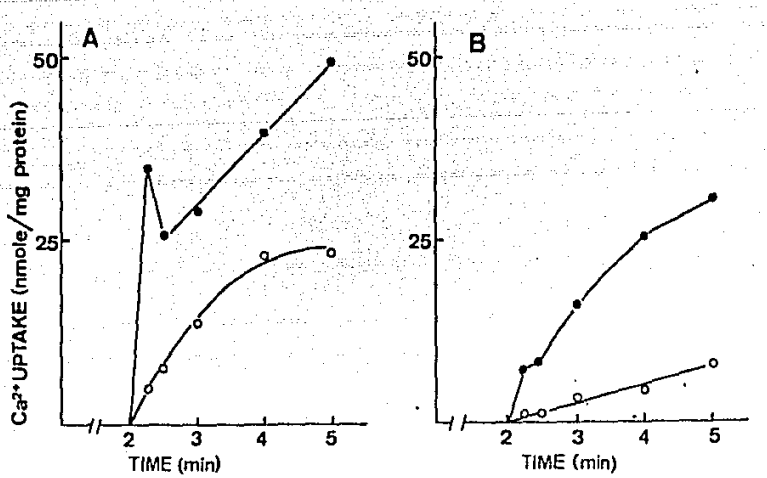

Figure 1. Effect of the Preincubation with Oxidizable Substrates and of Respiratory Inhibitors on the ATP-Supported Ca^{2+} Uptake by Heart Mitochondria. Mitochondria from heart (1.8 mg protein), prepared by grinding the tissue with sand, were preincubated at 25°C in 3 ml of 0.25 M sucrose, 12.5 mM Tris-HCl, pH 7.4. The reaction media also contained 5 mM glutamate-5 mM malate (A, closed circles), 4 µg rotenone (A, open circles), 5 mM succinate and 4 µg rotenone (B, closed circles), and 2 µg antimycin and 4 µg rotenone (B, open circles). After 2 min or preincubation, 300 µM $^{45}CaCl_2$ and 3 mM ATP were added to all reaction vessels. Together with Ca^{2+} and ATP, 4 µg rotenone (A, closed circles) or 2 µg antimycin A (B, closed circles) were added. At the indicated times aliquots were withdrawn, filtered through Millipore filters, washed, and counted in a scintillation counter.

The recent suggestion that the mitochondrial ATPase may exist in "inactive" and "active" states (2) determined by the transmembrane electrochemical gradient, was considered as a likely explanation for the Ca^{2+} uptake results shown in Figure 1. The transmembrane electrical potential was therefore measured directly with a tetraphenylphosphonium-sensitive electrode upon addition of ATP to liver mitochondria that had been preincubated with either succinate, or antimycin A. The traces in Figure 2 show very clearly that ATP polarizes the membrane to a final value of about 170 mv (negative inside) when added to succinate-preincubated mitochondria, but only to a final value of about 130 mv in the case of antimycin A preincubation. Thus, the preincubation under deenergizaing conditions indeed results in the transition of the ATPase towards a more inactive form. The alternative possibility was also considered that the preincubation under de-energizing conditions resulted in a decreased activity of the adenine-nucleotides translocase, which is known to be partly electrogenic (6). However, direct tests of the

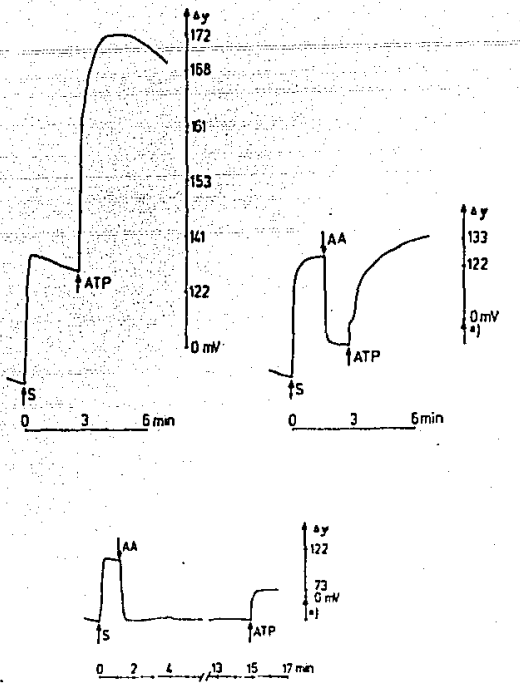

Figure 2. Transmembrane potential developed by ATP in liver mitochondria incubated with succinate or with antimycin A. Mitochondria (0.5 mg of protein per ml) were incubated at 25° in 0.13 M KCl, 10 mM Hepes buffer, pH 7.4, and 2 µg rotenone per mg of protein. At point "S" 1 mM succinate was added, at point ATP, 0.5 mM ATP, at point AA, 1 µg antimycin A per mg or protein. The electrode response was calibrated as indicated in (5). The lower trace in the Figure is a calibration for an apparently unspecific effect of ATP, which is visible also in the upper traces, and was, accordingly, subracted. Fifteen min of incubation in the presence of antimycin A evidently abolished completely the proton motive force, thereby inhibiting the penetraion of ATP.

translocase have shown it to be unaffected by the experimental conditions of Figures 1 and 2 (F. Alatriste et al., unpublished).

It follows from the previous data that the hydrolytic activity of the ATPase could show changes that parallel the capacity of mitochondria to accumulate Ca^{2+}. Figure 3 shows that the changes that occur as a function of time in Ca^{2+} uptake and ATPase activity in mitochondria exposed to either antimycin or succinate are indeed parallel. Antimycin preincubation induces a dramatic decrease in both the capacity of mitochondria to accumulate Ca^{2+} and their ability to hydrolyze ATP.

On the other hand, in mitochondria preincubated with succinate, both activities are considerably higher. It should be noted that the effects of antimycin occur within seconds of its addition.

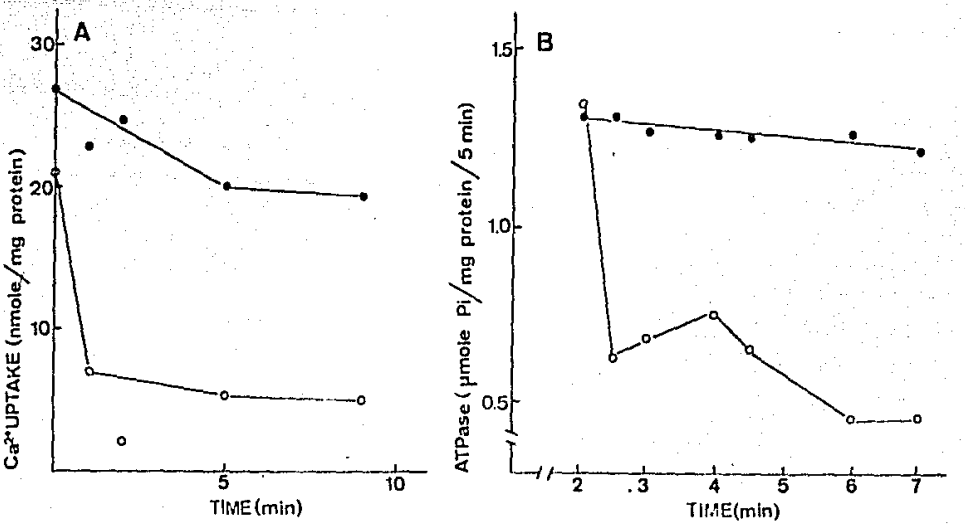

Figure 3. Effect of the Time of Preincubation with Oxidizable Substrates or Antimycin on the ATP-Supported Ca^{2+} Uptake, and on the ATPase Activity of Heart Mitochondria. Mitochondria were prepared by the Method of Vercesi et al. (4). In A, 0.6 mg of mitochondrial protein were preincubated at 25°C in 1 ml of 0.25 M sucrose, 15 mM Tris-HCl, pH 7.4 and 4 µg rotenone. The reaction medium also contained 5 mM succinate (closed circles) or 2 µg antimycin (open circles). At the times indicated in the Figure, mitochondria were transferred to reaction vessels containing 100 nmoles $^{45}CaCl_2$, 3 mM ATP and (closed circles) 2 µg antimycin. After two min of incubation, aliquots were withdrawn, filtered, washed, and counted. In B, mitochondria (10 mg protein) were preincubated for two minutes in 7 ml of 0.25 M sucrose, 12.5 mM Tris-HCl pH 7.4, 5 mM succinate and 15 µg rotenone. At the times shown, 0.5 ml aliquots were withdrawn and added to 0.5 ml of distilled water that contained 3 mM ATP and 1 µM FCCP, and the amount of inorganic phosphate liberated in 5 min was assayed. After the first aliquot (2 min of incubation) was withdrawn, 15 µg antimycin was added to one incubation flasks (open circles), samples were taken thereafter, and their ATPase activity was determined.

The data presented in Table II show that the higher ATP-supported Ca^{2+} uptake that is observed in mitochondria exposed to succinate is inhibited by atractyloside and oligomycin. The former prevents the influx of ATP into the mitochondria (6), while the latter inhibits the hydrolysis of ATP by interfering with the translocation of H^+ which is obligatorily coupled to the hydrolysis of ATP (7). Therefore, the hydrolysis of ATP is indeed the factor that accounts for the higher ability of mitochondria to accumulate Ca^{2+} when exposed to oxidizable substrates. Table II also shows that the inhibitor of Ca^{2+} transport, ruthenium red (8,9) and the uncoupler FCCP, block the ATP-supported influx of Ca^{2+} into mitochondria.

TABLE II

Effect of Atractyloside, Oligomycin, Ruthenium Red and FCCP of the ATP-supported Ca^{2+} Uptake by Heart Mitochondria.

Inhibitor present in the preincubation mixture	Preincubation with antimycin	Preincubation with succinate
	Ca^{2+} Uptake (nmole per mg protein)	
--	15.2	69.5
Atractyloside	2.0	3.5
Oligomycin	3.2	2.2
Ruthenium red	2.2	2.5
FCCP	3.0	2.2

Heart mitochondria were incubated as in the experiment of Table I, with 2 µg antimycin or 5 mM succinate. The preincubation mixture also contained 50 µM atractyloside, 5 µM ruthenium red, 2 µg oligomycin, or 1 µM FCCP, as indicated. Ca^{2+} uptake was initiated by the addition of $^{45}CaCl_2$ (100 nmole), 3 mM ATP, and 2 µg antimycin. Three minutes later an aliquot was filtered through Millipore filters, washed and counted in a scintillation counter.

The effects of substrate preincubation on the ATP-supported Ca^{2+} uptake are observed also when mitochondria are exposed to relatively low concentrations of Ca^{2+}. Figure 4 shows that the uptake is higher in mitochondria preincubated with succinate even when the concentration of added Ca^{2+} is of the order of 10 µM.

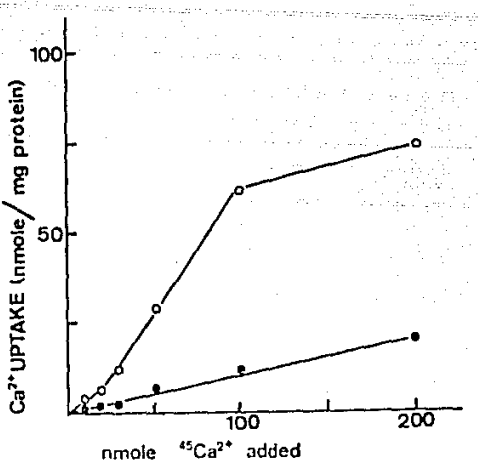

Figure 4. Ca^{2+} Uptake at Various Concentrations of added Ca^{2+} by Heart Mitochondria preincubated with Antimycin or Succinate. Mitochondria: (0.6 mg protein) prepared by the method of Vercesi et al. (4) were incubated for 6 min at 25°C in 1 ml of 0.25 M sucrose, 15 mM Tris-HCl, pH 7.4 and 4 µg rotenone. The incubation mixture also contained 2 µg antimycin (closed circles) or 5 mM succinate (open circles). At 6 min Ca^{2+} uptake was initiated by the simultaneous addition of $^{45}CaCl_2$ (at the concentrations shown) and 3 mM ATP to the antimycin treated mitochondria and by $^{45}CaCl_2$, 3 mM ATP and 2 µg antimycin to the succinate treated mitochondria. After three minutes the different mixtures were filtered through Millipore filtres, washed and counted.

ATP-Supported Ca^{2+} Uptake in Liver Mitochondria

The electrochemical gradient-dependent regulation of mitochondrial ATPase by the inhibitory peptide isolated by Pullman and Monroy (3), has only been described in heart mitochondria (2), and chloroplasts (10). To test whether the phenomena so far described have general significance, the ATP-supported Ca^{2+} uptake of liver mitochondria was studied. Figure 5 illustrates that liver mitochondria preincubated with succinate also exhibit a higher capacity to accumulate Ca^{2+} in the system supported by ATP, as compared to mitochondria in which the development of electrochemical gradients was prevented by antimycin. The Figure also shows that the level of the ATP-supported uptake of Ca^{2+} correlates with that of the ATPase activity.

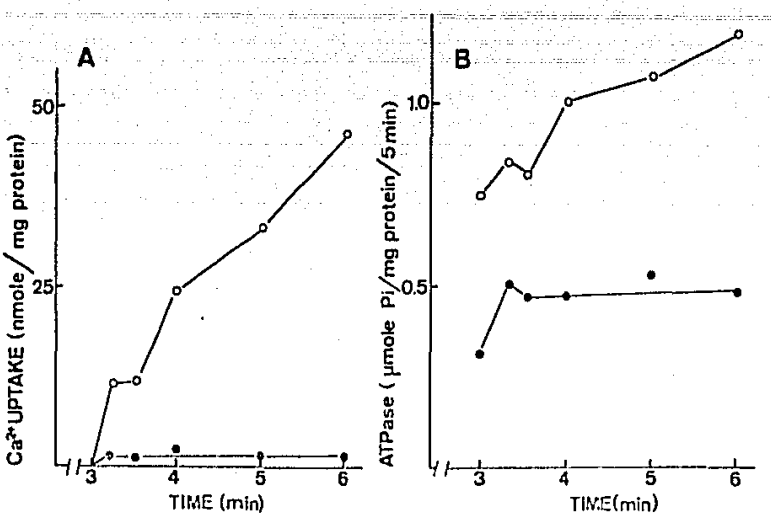

Figure 5. Ca^{2+} Uptake and ATPase Activity of Liver Mitochondria preincubated with Succinate or Antimycin. Mitochondria (8 mg of protein) were preincubated at 25°C in 10 ml of 0.25 M sucrose, 12.5 mM Tris-HCl, pH 7.4, and 15 µg rotenone. The incubation mixture also contained 5 mM succinate (open circles) or 15 µg antimycin (closed circles). After 3 min 100 µM $^{45}CaCl_2$ and 3 mM ATP (A, closed circles) and 100 µM $^{45}CaCl_2$, 3 mM ATP and 15 µg antimycin (A, open circles) were added. At the indicated times, aliquots were withdrawn, filtered through Millipore filters, washed and counted. For measurements of ATPase activity, aliquots of 0.5 ml were also withdrawn from the reaction medium and added to 0.5 ml of water that contained 3 mM ATP and 1 µM FCCP. After 5 additional minutes the reaction was stopped and the inorganic phosphate liberated assayed.

DISCUSSION

The electrochemical gradient derived from ATP hydrolysis is the driving force for the ATP-driven, ruthenium red-sensitive, movement of Ca^{2+} across the inner membrane (1). Therefore, the rate of Ca^{2+} influx in this system depends on the activity of the oligomycin sensitive ATPase. As a consequence, situations that "control" the ATPase activity would result in an indirect modulation of the rate of Ca^{2+} influx into mitochondria. The experiments described here have confirmed that the activity of mitochondrial ATPase may indeed be controlled by electrochemical gradients derived from electron transport. The behaviour

of the ATPase activity in intact mitochondria is thus analogous to that previously observed in heart submitochondrial particles, where it was shown (2), that the changes of activity induced by electrochemical gradients are mediated by the inhibitory peptide of Pullman and Monroy (3). The evidence presented here indicates that the rate of ATP-supported Ca^{2+} uptake is apparently controlled by the variations in activity of the ATPase system. Since the reconstitution studies mentioned above (2) have shown that these variations depend on the shifting of the inhibitory peptide to and from the ATPase, it follows that the ability of mitochondria to transport Ca^{2+} from their external milieu to their matrix compartment also depend on this shifting.

It should be noted, however, that in the regulation of Ca^{2+} uptake rates, other factors are likely to be involved, in addition to the inhibitory peptide. For example, it may be observed that the amount of ATP hydrolyzed, even under conditions in which the ATPase is in the "inactive" state, is much larger on a molar basis than the amount of Ca^{2+} accumulated under conditions of "active" ATPase, (Figs. 2 and 5). It may also be observed that even at very low concentrations of added Ca^{2+} (Fig. 4), the uptake is faster when the mitochondria had been previously exposed to electrochemical gradients. These observations suggest that other voltage-activated components may be involved in the regulation of the Ca^{2+} uptake.

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D

CAPITULO V

DISCUSION GENERAL

EL MMIQ Y EL K^+ COMO EFECTORES DEL SITIO I

En la sección A del Capítulo II reportamos que el MMIQ es un compuesto que interfiere con el transporte de electrones mediante una acción incidente en el segmento NADH-CoQ. Esta conclusión surgió de la alteración de varios parámetros: la transición del estado 4- estado 3 se inhibía y la respiración inducida por DNP se inhibía más apreciablemente cuando los sustratos respiratorios eran glutamato-malato y no succinato. Probablemente con respecto al último pueda existir un ligero efecto de inhibición en su entrada, no obstante, este hecho no afectaría la interpretación de la inhibición del MMIQ sobre el sitio I de la cadena respiratoria, dados los resultados obtenidos con partículas submitocondriales.

Las características de la estructura molecular del inhibidor sin duda le confieren efectos especiales en su modo de acción, afectando su afinidad por el sitio de inhibición en el complejo, su estabilidad y su número de blancos en la membrana; esto podría explicar que la concentración de MMIQ necesaria para inhibir la respiración sea superior a la que se utiliza al emplear rotenona o piericidina.

La exploración mas a fondo del sitio específico en la NADH deshidrogenasa donde interactúa el MMIQ y todos los inhibidores respiratorios daría información muy valiosa sobre la estructura y el mecanismo de los complejos de la cadena de transporte de electrones, ya que la topología que observan en la membrana es poco conocida. En cuanto a los mecanismos que rigen las actividades óxido-reductora de los complejos y de transferencia de protones, tanto los inhibidores del transporte de electrones como otros inhibidores de grupos químicos específicos pueden dar luz sobre la cuestión, de hecho el uso de reactivos como el DCCD (125,126,127) están aportando evidencias muy importantes en

favor de que los componentes del sistema de transportes del sistema de transporte de electrones pueden funcionar como bombas de protones por un mecanismo diferente al propuesto por Mitchell (128,129).

Podemos mencionar algunas de las muchas preguntas por ser contestadas sobre el sistema de transporte de electrones, acerca de la estructura:

¿Cuál es la topología de los complejos en la membrana, la de los componentes intra-complejo, la estequiometría de los complejos entre sí y su distribución en la membrana?

Y acerca de la mecanística del proceso:

¿Cómo es el acoplamiento entre la transferencia de electrones y la de H^+ dentro del mismo complejo y cuáles son las partes del mismo encargadas de cada proceso? Mediante que mecanismos moleculares se llevan a cabo? ¿Qué tanto pueden contribuir los cambios conformacionales de las porciones embebidas en la membrana? ¿Son de un mismo tipo los mecanismos de transferencia en los cuatro complejos respiratorios? ¿A qué profundidad de la membrana se encuentran los sitios donde se efectúan las transferencias?

Sobre la acción del K^+ en las mitocondrias, en la sección B del capítulo II presentamos las evidencias para considerarlo como un agente estimulante del sitio I y de la actividad hidrolítica de la ATPasa. Este efecto del K^+ puede conducir a consideraciones de tipo regulatorio, ya que las fluctuaciones en los niveles de K^+ intramitocondrial pueden conducir a fluctuaciones en las actividades de los complejos I y V, si bien alrededor existen cuestiones de estructura y mecanismo. Es interesante que un catión tan abundante tenga solo dos blancos de acción entre muchos posibles, que en ambos sea estimulatorio y que ambos pertenezcan a dos componentes tan relacionados funcionalmente.

Además otros cationes como el Li^+, Na^+ y Cs^+ carecen de estas propiedades (59). Curiosamente el Rb^+ se comporta igual que el K^+, lo cual habla de una especificidad en radio iónico bastante estricta por parte del sitio receptor de la proteína al K^+. Es posible que el impacto del catión induzca una conformación diferente en la enzima que se traduzca en un aumento de actividad hidrolítica, quizás a través de un aumento en la afinidad de la F_1 por su sustrato. Aunque hay un aumento en la velocidad de la catálisis según se muestra en la gráfica de dobles recíprocas, sería adecuado averiguar más sobre las constantes cinéticas que nos permitieran construir y afinar una explicación, ya que los datos bien podrían encajar con la desaparición del sitio de baja afinidad por la interacción del K^+ con la proteína. De cualquier manera esto podría ser compatible o reconciliable con un esquema de efecto conformacional por el K^+.

Se han reportado dos posibles explicaciones de la estimulación del K^+ en el segmento NADH deshidrogenasa - CoQ. Una de ellas propone que el K^+ funciona modificando la carga de superficie de la región activa de la NADH-DH, disminuyendo su afinidad por el NAD^+, el cual es el producto de la catálisis del complejo y que tiene un efecto inhibidor sobre el sistema (130). Sin embargo los datos de Cárabez y Sandoval sugieren fuertemente que el catión puede tener una acción estimulatoria más específica interactuando con la NADH deshidrogenasa en sitios para cationes (131), ya que el poliéter XXVIII que es un quelante de K^+, Rb^+ y Cs^+, inhibe la oxidación de $NADH^+$.

Resultaría muy interesante saber bajo que condiciones fisiológicas sería operante el proceso de stimulación de la NADH y de la ATPasa por K^+, así como de su contribución a la regulación del metabolismo energético. Tal vez

ahora sea posible introducir un nuevo modulador de la fosforilación oxidativa.

LAS INTERACCIONES HIDROFOBICAS Y LOS CAMBIOS CONFORMACIONALES EN LA ATPasa MITOCONDRIAL.

Acerca de la conformación de la enzima, con el óxido de deuterio encontramos un arma poderosa para expresar en forma amplificada las fuerzas hidrofóbicas que operan entre las subunidades de la ATPasa, y en su interacción con la bicapa y el solvente, esto está expresado en el trabajo de la sección A del capítulo III. Los resultados evidenciaron una protección de la enzima por el agua deuterada a la inactivación por frío y la aparente rigidización de la enzima al presentar una actividad de hidrólisis muy poco sensible a la estimulación por K^+ y HCO_3^- y a la inhibitoria de la octilguanidina. La medición del incremento en la fluorescencia del ANS parece reforzar la interpretación de que estos efectos se deben al aumento en la repulsión de los grupos polares hacia regiones hidrofílicas. Sin embargo permanece por determinarse la magnitud de las contribuciones de los grupos apolares que están en los confines de la enzima y de los que se hallan en las demarcaciones entre una y otra subunidad.

Cabe también considerar la posibilidad de que la actividad hidrolítica de la ATPasa se vea modificada por el D_2O no sólo a través de efectos sobre las interacciones hidrofóbicas que trascienden a la conformación de la enzima, sino también por un efecto isotópico del D_2O, ya que la hidrólisis del ATP involucra al H_2O como un reactivo de la reacción. Desgraciadamente se antoja difícil disecar si se trata de los dos efectos, o uno solo y cual puede ser la contribución de cada uno.

Existe mucha elucubración e interés en lo que se refiere a la forma que guarda la ATPasa en la membrana. Anteriormente se señaló que la enzima tiené porciones expuestas directamente a la matriz mitocondrial (24), pero qué tanto representan estas del cuerpo de la enzima y cuales son las que permanecen inmersas en la bicapa, son incógnitas por resolver. Por ello resulta de especial interés lo que se observa en las micrografías al microscopio electrónico de la sección A del Cap. III y que nos llevaron a dar cabida a la posibilidad de que en D_2O las interacciones hidrofóbicas enzima-membrana se hubieran acentuado provocando una extrusión más lenta de la enzima hacia la fase acuosa, cosa que aparentemente sucede rápidamente cuando el choque hipotónico se produce en H_2O.

Es indiscutible la relevancia que tiene el conocimiento de la relación de los grupos, fuerzas y dinámica de la periferia de la proteína con su entorno lipídico, ya que se dispondría de mas argumentos para ir conformando mecanismos catalíticos y de regulación de una forma más aproximada a lo que ocurre in situ.

Otra forma de explorar la modificación de las interacciones entre los diferentes grupos de la enzima durante la catálisis, es el enfoque cinético. Con esta idea diseñamos los experimentos de la sección B capítulo III, que nos llevaron a incluir octilguanidina y metanol como agentes que aumentan las interacciones hidrofóbicas. Sus efectos pudeiron ser comparados sobre el patrón bifásico de la actividad hidrolítica de ATP cuando se grafica según Lineweaver-Burk. Aparentemente con metanol solo hay una estimulación a temperaturas mayores o iguales a 30°C, lo cual podría interpretarse como un rejuego de los grupos de la enzima, inducido por cambios en hidrofobicidad, interac

ciones electrostáticas, etc. y que van siendo traducidos en cambios conformacionales de la proteína, lo cual puede determinar la participación de determinado sitio en la catálisis.

En este trabajo se postula la existencia de dos estados conformacionales de la proteína, según su sensibilidad a la temperatura, al metanol y a la octilguanidina y puede ser compatible con una segunda alternativa que incluye la presencia de dos sitios catalíticos, o bien de una tercera que introduce al metanol actuando en una reacción limitante del proceso de hidrólisis de ATP, disminuyendo la velocidad de salida de uno de los productos, el ADP (83)

Sobre este tema se pueden establecer muchas de las interrogantes por ser contestadas:

a) El acoplamiento de la F_1 al canal de protones, la transmisión del H^+ desde la F_o hasta el sitio donde se verifica la catálisis, el papel del H^+ en la síntesis de ATP, el papel de los sitios de enlace de nucleótidos y el mecanismo catalítico.

b) Describir los cambios conformacionales, entendiendo como tales cuáles son los residuos de los aminoácidos activos en la reacción catalítica, cuáles se ocultan, que porciones de las cadenas polipeptídicas se pliegan o se repliegan, cuáles son las zonas de la proteína que tienen mayor o menor movilidad. Hasta ahora hablar de cambios conformacionales en proteínas es sólo la manifestación del cambio, o las repercusiones de éste sobre parámetros de unión o actividad de algún ligando.

REGULACION DE LA ATPasa MITOCONDRIAL

En la Sección A del Capítulo IV, mostramos que los resultados que habían sido obtenidos por los grupos de Gómez-Puyou y Harris en partículas submito-

condriales eran también reproducidos en mitocondrias intactas, esto es, que la ATPasa puede existir en una forma activa o una inactiva, dependiendo de que el inhibidor haya sido disociado o no por el $\Delta_\mu H^+$.

En nuestro trabajo los datos mas interesantes fueron la presencia de fluctuaciones de la actividad de ATPasa en respuesta a inhibiciones en diferentes sitios de la cadena de transporte de electrones y la inactivación de las ATPasas en condiciones en que se lleva a cabo el transporte de Ca^{++}. Este último hecho establecía una nueva forma, aunque indirecta, de regulación por parte de la proteína inhibidora con respecto a uno de los procesos mitocondriales más estudiados como es el transporte de Ca^{++}. La explicación que ofrecemos consiste en un papel asociador del Ca^{++} entre proteína inhibidora y F_1, aunque no descartamos la competencia de los procesos de disociación del inhibidor y el transporte de calcio, ya que ambos son mediados por el gradiente electroquímico.

Cuando exploramos más a fondo la relación entre el transporte de calcio y la activación de la ATPasa vía remoción del inhibidor, que se describe en la sección B del Capítulo IV, enconetramos una dependencia de la magnitud de la entrada de Ca^{++} y la actividad de ATPasa, cuando para el primero la fuerza impulsora es la hidrólisis de ATP y la segunda es manipulada a través de la utilización de sustratos oxidables o de inhibidores respiratorios que permitían o no el establecimiento de un $\Delta_\mu H^+$.

Este fenómeno manifiesta una modulación de la entrada de calcio cuando la fuerza impulsora es el potencial generado por la hidrólisis de ATP constituyendo una forma de regulación no descrita hasta ahora. Sin embargo este hallazgo debe ser tomado con reservas pues si se considera que la hidrólisis de

ATP bien puede no ser una función in situ, la relevancia de un mecanismo como el que aquí exponemos podría ser escasa; no obstante, la posibilidad de que el sistema ATP sintetasa tuviera un efecto sobre el transporte de calcio a través de la disociación del inhibidor no sería remota y valdría la pena de ser considerada, ya que se tienen antecedentes de que el transporte de calcio es una función que se efectúa prioritariamente a la síntesis de ATP, cuando prevalecen condiciones para ambos (132,133).

El hecho de que no se pueda aclarar si la dirección hidrolítica de la ATPasa in vivo existe, puede ser relevante al papel de la proteína inhibidora. Como indicamos anteriormente esta proteína había sido propuesta como un inhibidor undireccional, inhibiendo la hidrólisis y no la síntesis, posteriormente quedó demostrada su acción inhbiendo los estados iniciales de la fosforilación oxidativa. Cabe remarcar aquí que el efecto de la proteína inhibidora parece ser diferente tanto cuantitativa como cualitativamente en la hidrólisis y en la síntesis de ATP. ¿Es entonces la función del inhibidor, inhibir la catálisis en dirección de la hidrólisis y modularla en la dirección de la síntesis? Al respecto Harris (134) ha propuesto un modelo ek el que el inhibidor se asocia y disocia en un equilibrio dinámico dando una población de moléculas de ATPasa que sintetizan ATP.

Con cada nuevo dato que surge acerca del funcionamiento del inhibidor, se van planteando y replanteando cuestionamientos importantes que una vez resueltos deben contribuir al entendimiento de los mecanismos que regulan la actividad de la ATPasa.

Entre los puntos más importantes por contestar, señalo los siguientes:

a) Dilucidar cual es el mecanismo molecular a través del cual el inhibidor o la ATPasa "detecta" el potencial generado o la ausencia del mismo.

b) Establecer si el llamado desplazamiento del inhibidor bajo el efecto del $\Delta\mu H^+$, corresponde a un cambio de conformación de la molécula, a un verdadero cambio del lugar físico de unión a la OS-ATPasa o a una liberación del inhibidor hacia el medio. En el caso de la existencia de un segundo sitio se abrirían posibilidades muy interesantes.

c) Describir cuales son los factores que determinan el regreso del inhibidor a su sitio o a su forma inhibitoria.

d) Explorar, dentro de los eventos que conforman la catálisis, cuál es el momento en que el inhibidor deja de ejercer acción, y que tan directa o indirectamente interactúa con el sitio catalítico, así como el funcionamiento del inhibidor en los modelos que describen la síntesis de ATP y en especial en el de Adolfsen y Moudrianakis, el cual es llamado modelo de los sitios alternantes (136), en el que se postulan 2 ó 3 sitios catalíticos de acuerdo con el número de subunidades catalíticas y que funcionan relevándose.

e) Determinar cuantas moléculas de ATP pueden sintetizarse por una molécula de ATPasa en el tiempo en el que el inhibidor está en su sitio no-inhibitorio. Esto daría información sobre la eficiencia del sistema y la velocidad del mecanismo enzimático. Además, proporcionaría indicios para esclarecer los siguientes puntos.

f) Aclarar que está pasando con el inhibidor en la población total de enzimas (de la cual sólo un 10% es activa), cuando se observa el retardo experimental de la fosforilación.

g) Mostrar cual es la dinámica de "encendido" y "apagado" que sigue la población de ATPasas bajo el establecimiento de $\Delta_{\mu}H^{+}$. Es evidente que metodológicamente no resulta fácil resolver este punto.

En 1931 Engelhardt encontró el acoplamiento entre la respiración y la fosforilación iniciando una época en la que predominaron las respuestas a los fenómenos que empezaron a caracterizarse, curiosamente no siempre se tenía mucha idea en el planteamiento de las preguntas, ya que la investigación se enfrentaba a sistemas desconocidos. 50 años después la situación ha cambiado: se tiene mucha idea de lo que hay que preguntar, pero ahora no parece fácil encontrar la forma de contestar las nuevas preguntas. Con la ATPasa y su proteína inhibidora tenemos cada vez más interrogantes y las respuestas están surgiendo continuamente. Tal vez sea necesario un cambio de estrategias para responder a cuestionamientos más refinados en la estructura y la función de las ATPasas de protones y de su regulación; probablemente los sistemas modelo y la biología molecular sean las herramientas más prometedoras por el momento para disecar efectos y propiedades. Sin embargo para comprender el funcionamiento de la ATP sintetasa como un todo integrado a su ambiente, será inevitable regresar al sistema mitocondrial en el que se realizan concertadamente todos los procesos que integran su fisiología.

CAPITULO VI

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