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Identification by Site-directed Mutagenesis of Three Arginines in Uncoupling Protein That Are Essential for Nucleotide Binding and Inhibition*

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Martin Modrianský‡§, Debra L. Murdza-Inglis¶, Hasmukh V. Patel¶, Karl B. Freeman¶, and Keith D. Garlid‡‖

From the ‡Department of Biochemistry and Molecular Biology, Oregon Graduate Institute of Science and Technology, Portland, Oregon 97291-1000 and the ¶Department of Biochemistry, McMaster University, Hamilton, Ontario L8N 3Z5, Canada

Primary regulation of uncoupling protein is mediated by purine nucleotides, which bind to the protein and allosterically inhibit fatty acid-induced proton transport. To gain increased understanding of nucleotide regulation, we evaluated the role of basic amino acid residues using site-directed mutagenesis. Mutant and wild-type proteins were expressed in yeast, purified, and reconstituted into liposomes. We studied nucleotide binding as well as inhibition of fatty acid-induced proton transport in wild-type and six mutant uncoupling proteins. None of the mutations interfered with proton transport. Two lysine mutants and a histidine mutant had no effect on nucleotide binding or inhibition. Arg^{83} and Arg^{182} mutants completely lost both the ability to bind nucleotides and nucleotide inhibition. Surprisingly, the Arg^{276} mutant exhibited normal nucleotide binding, but completely lost nucleotide inhibition. To account for this dissociation between binding and inhibition, we propose a three-stage binding-conformational change model of nucleotide regulation of uncoupling protein. We have now identified three nucleotides by site-directed mutagenesis that are essential for nucleotide interaction with uncoupling protein.

Uncoupling protein (UCP)[1] from brown adipose tissue mitochondria mediates proton flux from cytosol back into the matrix, thereby producing heat (1, 2). UCP contains an anion transport pathway that permits the charged fatty acid anion head group, to which the membrane is normally impermeable, to flip-flop in the membrane. The cycle is completed by flip-flop of the protonated fatty acid head group in the bilayer, resulting in energy-dissipating back flux of protons into the matrix (3). The only known regulation of the uncoupling cycle is provided by purine nucleotides, which bind to UCP and allosterically inhibit transport (4).

UCP does not contain a consensus nucleotide binding sequence, and relatively little is known about the location of the nucleotide binding site. Klingenberg's laboratory (5, 6) has shown by chemical labeling that the base and ribose moieties are positioned near the matrix loop connecting the fifth and sixth transmembrane domains. Lysine and arginine residues have long been suspected of participation in nucleotide regulation, and Katiyar and Shrago (7) showed that phenylglyoxal abolished GDP binding to UCP. However, none of the residues that interact with the phosphate groups have been identified.

The introduction of a high-level expression system for UCP in *Saccharomyces cerevisiae* (8) opened the door to a study of these interactions using site-directed mutagenesis. Thus, we were able to show that mutation of a single arginine residue (Arg^{276}) abolished GDP inhibition without affecting UCP-mediated proton transport (9). We have now extended this study to Arg^{83}, Arg^{182}, Lys^{268} and His^{214}, which are positioned within the membrane-spanning helices of UCP, and Lys^{72}, which lies outside the transmembrane domain (10). The mutant proteins were overexpressed in yeast (8, 9), then purified and reconstituted into liposomes. Fatty acid-induced, UCP-mediated H^+ transport, and its sensitivity to nucleotides, were assayed by fluorescence, and GDP binding was assayed using [^{3}H]GDP.

Each of the three arginine mutations caused complete loss of GDP inhibition of transport without affecting transport itself. This finding implies an extensive nucleotide interaction domain, because the three arginines are located on three different transmembrane segments. A surprising finding was that the Arg^{276} mutant, despite complete loss of sensitivity to GDP inhibition (9), retained normal binding affinity for GDP. We propose a three-stage binding-conformational change model in which Arg^{276} interacts with nucleotide after it is tightly bound, causing a conformational change that prevents transport of the fatty acid anion head group.

EXPERIMENTAL PROCEDURES

Materials—Zymolyase 100T was purchased from Seikagaku America (Ijamsville, MD). [^{3}H]GDP was purchased from NEN Life Science Products. Dowex 1-X8, 200–400 mesh anion exchanger (chloride form) was obtained from Sigma. All other materials for cloning, site-directed mutagenesis, yeast growth, yeast mitochondria isolation, UCP isolation, and reconstitution were from sources listed previously (9).

Site-directed Mutagenesis—A M13mp19 plasmid containing the rat UCP cDNA fragment was used for preparing the site-directed mutants by the method of Kunkel (11). Oligonucleotides were designed to alter UCP codons for Arg^{83} (AGG) to Gln (CAG), Arg^{182} (AGA) to Thr (ACA), Lys^{72} (AAA) to Gln (CAA), Lys^{268} (AAA) to Gln (CAA), and His^{214} (CAT) to Gln (CAA). The *Sac*I/*Sph*I fragments from M13mp19 containing wild-type or mutated forms of UCP cDNA were subcloned into *Sac*I/*Sph*I-cut pCGS110 *Escherichia coli*/*S. cerevisiae* shuttle vector. The cDNAs are under transcriptional control of the inducible *Gal*I promoter. The *S. cerevisiae* strain JB516 (*MATa*, *ura*3, *ade*1, *leu*2, *his*4, *gal*$^+$) was transformed by electroporation with the shuttle vector constructs and plated on uracil-lacking selective plates. The resulting yeast transformants were grown at 30 °C in selective medium, and overexpression of UCP was induced by addition of 0.2% galactose (8).

Purification and Reconstitution of Mutant UCPs—Yeast cells were

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‖ To whom correspondence and reprint requests should be addressed: Dept. of Biochemistry and Molecular Biology, Oregon Graduate Institute, P. O. Box 91000, Portland, OR 97291-1000. Tel.: 503-690-1680; Fax: 503-690-1464.

[1] The abbreviations used are: UCP, uncoupling protein; UCPH, uncoupling protein homolog; SPQ, 6-methoxy-*N*-(3-sulfopropyl)quinolinium; TES, *N*-tris(hydroxymethyl)methylaminoethane sulfonic acid; TEA, tetraethylammonium cation.

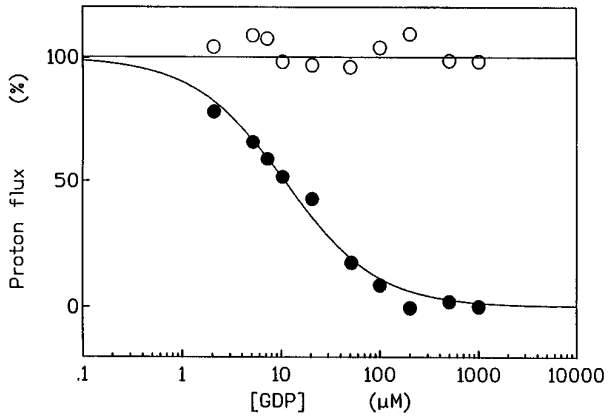

FIG. 1. **GDP inhibition of wild-type and $Arg^{182} \rightarrow Thr$ mutant UCPs.** Proton fluxes, as percent of control rate in the absence of external GDP, are plotted *versus* external [GDP]. The data shown were obtained with wild-type (●) and $Arg^{182} \rightarrow Thr$ (○) mutant in the presence of 30 μM laurate. The K_i for GDP inhibition of wild-type UCP was 11 μM. The difference between the maximum flux ("100% flux") and the minimum flux ("0% flux") corresponds to rates shown in Table I.

harvested after growth in galactose for 11 h, and yeast mitochondria were isolated using a protocol modified from Gasser (12). The final mitochondrial pellet was resuspended and stored at −20 °C in buffer consisting of 250 mM sucrose, 5 mM K-TES, pH 6.7, and 1 mM K-EGTA. UCP was extracted, purified, and reconstituted into liposomes using the protocols described in detail by Garlid *et al.* (13).

Assay of H^+ Transport Mediated by Mutant UCPs—SPQ fluorescence is quenched by TES anion, but not by TES zwitterion, allowing measurements of changes in total acid concentration (14). SPQ (2 mM) trapped inside liposomes containing the mutant UCPs was used for assaying transport and GDP sensitivity of the transport, as before (9). The intravesicular medium contained TES (28.8 mM), EGTA (0.6 mM), and SO_4 (84.4 mM), pH 7.2, all TEA salts, and 1 mM GDP. The external medium, also at pH 7.2, contained TES (28.8 mM), and EGTA (0.6 mM) as TEA salts, and SO_4 (84.4 mM) as K^+ salt. Proton efflux from the liposomes was initiated by adding 30 μM laurate followed by 0.1 μM valinomycin. Each proteoliposome preparation was calibrated to obtain the corresponding quench constant, and volume of the liposomes was estimated from the probe distribution. Protein content of liposomes was estimated by the Amido Black method (15).

Assay of GDP Binding to Mutant UCPs—We used a modified anion-exchange method developed by Klingenberg *et al.* (16). A Pasteur pipette tip was sealed with packed glass wool and then filled with 30 mg of wet Dowex 1-X8, 200–400 mesh (chloride form). 75 μl of sample containing 6–9 μg of UCP was applied on top of the resin. Experimental medium contained [^{3}H]GDP in concentrations from 1 to 15 μM and otherwise was the same composition as internal medium, described above. The sample was immediately chased with 2 × 100 μl of distilled H_2O through the column with a bulb. The entire eluate was collected and subjected to scintillation counting. For control, 100 μM nonradioactive GDP was included with each concentration of the radiolabeled nucleotide.

RESULTS

Effects of Arg and Lys Mutations on GDP Inhibition of Proton Transport—Fig. 1 contains a typical GDP concentration dependence for inhibition of wild-type UCP (*solid circles*) and $Arg^{182} \rightarrow Thr$ mutant UCP (*open circles*). As shown in Table I, each of the arginine mutations completely abolished GDP inhibition, whereas the lysine and histidine mutants retained full GDP sensitivity. The K_i for GDP inhibition of proton flux in wild-type and native UCP under these conditions is 11 μM (9) and was not affected in the mutants exhibiting GDP inhibition.

Upon reconstitution, UCP is distributed randomly in the bilayer (17). It transports anions in both directions, but 1 mM GDP is present in the liposomal interior, so that 50% of the wild-type UCP is always inhibited. Loss of GDP regulation should therefore free up both orientations of the protein and cause doubling of transport activity. This expectation was met

TABLE I
GDP inhibition of proton fluxes mediated by mutant and wild-type UCP

The table contains values for proton flux (± S.E.) in the presence and absence of 1 mM GDP (normal K_i = 11 μM (9)). Proton flux in proteoliposomes was induced by 30 μM laurate. Rates are corrected for proton leak, estimated at 28 ± 2 μM H^+/s (n = 30). This was also the level to which 300 μM undecanesulfonate, a competitive inhibitor of laurate-induced proton flux (3), reduced proton flux.

UCP	n	Proton flux		Percent inhibition
		$[GDP]_{out}$ = 0	$[GDP]_{out}$ = 1 mM	
		μmol H^+ min^{-1} mg^{-1}		
Wild-type	7	2.89 ± 0.13	0.24 ± 0.08	93
$Lys^{72} \rightarrow Gln$	5	2.33 ± 0.44	0.19 ± 0.08	92
$Lys^{268} \rightarrow Gln$	3	2.43 ± 0.22	0.16 ± 0.15	93
$His^{214} \rightarrow Gln$	4	2.33 ± 0.56	0.13 ± 0.16	94
$Arg^{83} \rightarrow Gln$	4	6.70 ± 0.38	6.66 ± 0.39	1
$Arg^{182} \rightarrow Thr$	5	3.23 ± 0.46	3.02 ± 0.45	7
$Arg^{276} \rightarrow Leu$	3	3.16 ± 0.59	3.14 ± 0.52	1

TABLE II
GDP binding to mutant and wild-type UCPs

Table contains the dissociation constants, K_d, and binding capacity, B_{max}, derived from Eadie-Scatchard plots for each of the mutants. The value for wild-type protein translates to 0.9 mol of GDP/mol of UCP, assuming that one-half of the sites are available for binding due to random insertion. This stoichiometry is in agreement with that of Rafael *et al.* (20).

Uncoupling protein	K_d	B_{max}
	μM	*nmol/mg protein*
Wild-type	7.2	15.1
$Lys^{72} \rightarrow Gln$	9.2	12.3
$Lys^{268} \rightarrow Gln$	7.2	10.6
$Arg^{83} \rightarrow Gln$	No binding	No binding
$Arg^{182} \rightarrow Thr$	No binding	No binding
$Arg^{276} \rightarrow Leu$	7.8	14.1

in the $Arg^{83} \rightarrow Gln$ mutant, but not in the other two Arg mutants.

Undecanesulfonate anion is transported by UCP and is a competitive inhibitor of laurate-induced proton flux (3). 300 μM undecanesulfonate inhibited proton flux in all mutants to the level of proton leak, consistent with normal behavior of the fatty acid anion transport pathway in the mutant UCPs.

Effects of Arg and Lys Mutations on GDP Binding—The concentration dependence of GDP binding yielded linear mass-action plots, from which K_d and B_{max} values were obtained. These parameters are contained in Table II. In comparing the data in Tables I and II, there is agreement between GDP binding and inhibition in each case except one. Thus, the $Arg^{83} \rightarrow Gln$ and $Arg^{182} \rightarrow Thr$ mutants lost both GDP inhibition and GDP binding, whereas the $Lys^{72} \rightarrow Gln$ and $Lys^{268} \rightarrow Gln$ mutants retained both functions. The $Arg^{276} \rightarrow Leu$ mutant is unique in that it binds GDP normally but cannot be inhibited by GDP. Retention of normal GDP binding by the $Arg^{276} \rightarrow Leu$ mutant has been confirmed in isolated yeast mitochondria (18). The protocol used to assay binding captures only the tightly bound state (19); thus, GDP is tightly bound in the Arg^{276} mutant.

The dissociation constants, K_d, of the mutant UCPs capable of binding GDP (Table II) are higher than published values pertaining to native rat UCP (20); however, the K_d values are close to the observed K_i for GDP inhibition of UCP-mediated transport, which is generally 11 μM (9). This agreement may reflect our use of identical conditions for H^+ flux and binding measurements.

Lysine 268 is located within the third domain of the tripartite structure among residues 238–283, which have long been identified with nucleotide interactions (6), and Lys^{72} is located

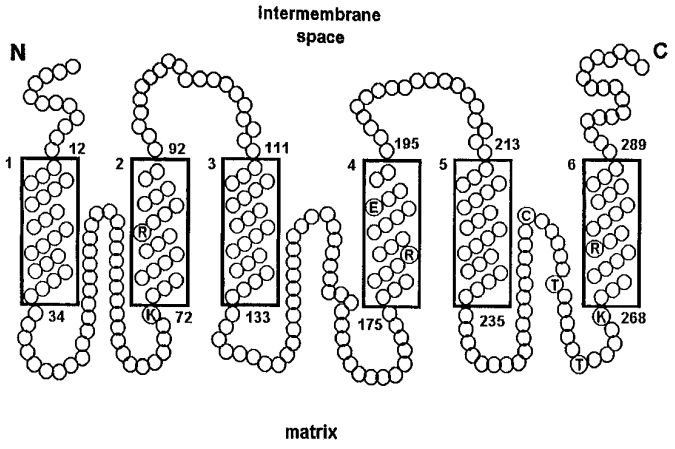

FIG. 2. **Membrane-spanning model of uncoupling protein.** UCP contains six transmembrane domains, which are designated by the single digit numbers. Membrane topology follows Miroux *et al.* (29). Amino acids at the cytosolic and matrix ends of the α-helices are numbered based on models of Klingenberg (1) and Winkler *et al.* (25). Lys^{72} and Lys^{268} were mutated without effect (this study). The remaining designated residues have been shown to participate in nucleotide binding to UCP. Thr^{259}, Thr^{264}, and Cys^{253} interact with the sugar-base moiety of the nucleotide (25). Arg^{83}, Arg^{182}, and Arg^{276} are essential for nucleotide binding and/or inhibition (this study), as is Glu^{190} (25). Each of the four transmembrane residues, Arg^{83}, Arg^{182}, Arg^{276}, and Glu^{190}, is conserved in UCPH, as is Cys^{253}. UCPH contains substitutions at position 259 (Gln instead of Thr) and 264 (Arg instead of Thr).

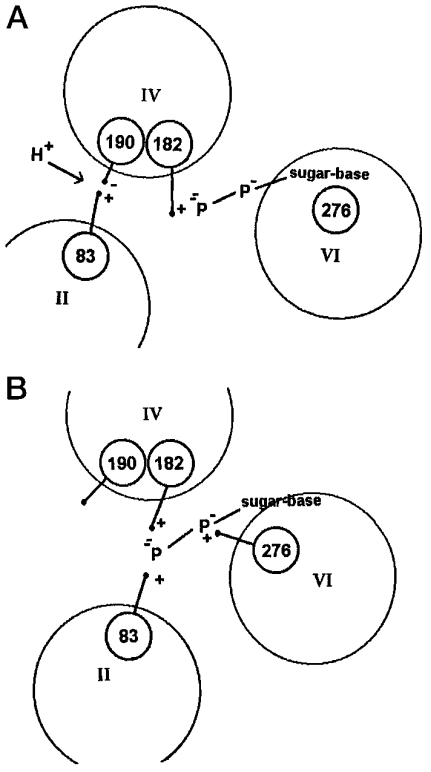

FIG. 3. **Model of the phosphate binding pocket in UCP.** *A*, loose conformation. The nucleotide sugar-base moiety binds loosely to the loop between transmembrane helices 5 and 6, and the terminal phosphate binds to Arg^{182} on helix 4. Binding to Arg^{83} is prevented by a salt bridge from Glu^{190} to a neighboring Arg or Lys. The figure tentatively identifies this residue as Arg^{83} itself. *B*, tight conformation/inhibited conformation. Protonation of Glu^{190} opens the phosphate binding cleft between helices 2 and 4 and frees Arg^{83} to bind to the terminal phosphate of nucleoside diphosphates, resulting in a tightly bound state. Tight binding pulls the bound nucleotide, together with its sugar-base attachments at the N terminus of helix 6, into a new position, causing a demonstrable conformational change at the C terminus of helix 6 (19). Tight binding is not sufficient to inhibit transport. This occurs when Arg^{276} binds to the α-phosphate, causing a further conformational change that occludes the intramembrane binding site for the anionic head group of fatty acids.

in the second domain. As shown in Tables I and II, neither residue affected GDP binding or GDP inhibition.

The Role of His^{214} in pH Regulation of Nucleotide Inhibition—The pH dependence of nucleoside triphosphate binding to UCP has a pK_a of 7.2, which suggested involvement of a histidine residue (21). If His^{214} plays this role, the $His^{214} \rightarrow Gln$ mutation should exhibit an increased K_i for GTP inhibition at neutral pH. In fact, this mutation had no effect on the K_i for GTP inhibition at pH 7.2 or 7.6 when compared with the wild-type (three experiments, data not shown).

DISCUSSION

Because of their polyanionic character, nucleotides are expected to bind to positively charged amino acid residues in UCP. Arg^{276} was the first such residue to be identified (9), and we now show that Arg^{83} and Arg^{182} are also essential for normal nucleotide binding and inhibition. None of the mutations affected H^+ transport, confirming that fatty acid anion transport and nucleotide inhibition are separate features of UCP and implying two distinct binding sites (4, 7).

Structural Implications for UCP and UCPH—The location of the three essential arginines implies an extensive interaction region involving at least three of the six transmembrane helices in UCP (Fig. 2). These helices very likely form the walls of a dead-end aqueous pocket for nucleotides, which are known to enter and exit from the cytosolic side and to interact with residues located on the matrix loop between helices 5 and 6 (6). Moreover, each of the three arginines is conserved in UCPH, suggesting that this protein is also regulated by nucleotides. UCPH, whose primary amino acid sequence is 56% identical with that of UCP, encodes a protein thought to be involved in prevention of obesity and diabetes (22, 23).

Proposed Three-stage Mechanism of Nucleotide Regulation—Klingenberg and co-workers (19, 21, 24, 25) have elaborated an elegant kinetic model of the interactions of nucleotide phosphates with UCP. Access to the phosphate binding region is controlled by protonation of Glu^{190}, presumably by releasing a salt bridge that occludes the binding site. Huang and Klingenberg (19) proposed a two-stage mechanism in which nucleotides are first loosely bound and then the protein undergoes a slow, spontaneous conformational change to a "tight" state in which nucleotides are tightly bound and H^+ transport is inhibited. Our results show, however, that the tightly bound state is not sufficient for transport inhibition. Thus, the tight binding exhibited by the Arg^{276} mutant is ineffective in inhibiting H^+ transport. Thus, the two-stage model (19) requires modifications, as described below and in Fig. 3.

Loose Conformation—The nucleotide enters the aqueous pocket in UCP, and its sugar-base moiety binds to the matrix loop connecting the fifth and sixth α-helices (5, 6). This interaction may be responsible for discrimination between purine and pyrimidine nucleotides (20, 26, 27). The nucleotide β-phosphate binds to Arg^{182}, and the sum of these interactions results in a loose binding conformation.

Tight Conformation—The switch to a tight binding conformation is initiated by protonation of Glu^{190}, making Arg^{83} available for binding to the second charge on the β-phosphate of diphosphates and the γ-phosphate of triphosphates. A histidine binds to the second charge on the γ-phosphate of triphosphates (24). The tightly bound conformation prevents trypsin cleavage of the C terminus of UCP at Lys^{292} (19). This step is necessary, but not sufficient, for inhibition of transport.

Inhibited Conformation—The nucleotide is now bound stably and positioned so that the α-phosphate can bind to Arg^{276}. This

final interaction induces a conformational change that occludes or removes the internal fatty acid binding site (3, 28), causing inhibition of transport. This extension of Huang and Klingenberg's model (19) is consistent with our finding that mutation of Arg^{276} to Leu did not affect the K_d for binding but completely prevented nucleotide inhibition of transport.

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