Site-Directed Mutagenesis Identifies Residues in Uncoupling Protein (UCP1) Involved in Three Different Functions†

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Received October 21, 1999; Revised Manuscript Received December 27, 1999

ABSTRACT: Using site-specific mutagenesis, we have constructed several mutants of uncoupling protein (UCP1) from brown adipose tissue to investigate the function of acidic side chains at positions 27, 167, 209, and 210 in H^+ and Cl^- transport as well as in nucleotide binding. The H^+ transport activity was measured with mitochondria and with reconstituted vesicles. These mutant UCPs (D27N, D27E, E167Q, D209N, D210N, and D209N + D210N) are expressed at near wt levels in yeast. Their H^+ transport activity in mitochondria correlates well with the reconstituted protein except for D27N (intrahelical), which shows strong inhibition of H^+ transport in the reconstituted system and only 50% decrease of uncoupled respiration in mitochondria. In the double adjacent acidic residues (between helix 4 and helix 5), mutation of D210 and of D209 decreases H^+ transport 80% and only 20%, respectively. These mutants retain full Cl^- transport activity. The results indicate that D210 participates in H^+ uptake at the cytosolic side and D27 in H^+ translocation through the membrane. Differently, E167Q has lost Cl^- transport activity but retains the ability to transport H^+. The separate inactivation of H^+ and Cl^- transport argues against the fatty acid anion transport mechanism of H^+ transport by UCP. The mutation of the double adjacent acidic residues (D209, D210) decreases pH dependency for only nucleoside triphosphate (NTP) but not diphosphate (NDP) binding. The results identify D209 and D210 in accordance with the previous model as those residues which control the location of H214 in the binding pocket, and thus contribute to the pH control of NTP but not of NDP binding.

The uncoupling protein (UCP1)[1] provides a regulated transport pathway for electrophoretic back-flux of H^+ into the mitochondrial matrix of brown adipose tissue, thereby dissipating proton motive force and producing heat (*1, 2*). H^+ transport is inhibited by purine nucleotides and activated by fatty acids (*3, 4, 15*). UCP1 also transports monovalent anions electrophoretically (*5, 6*). Recently, new uncoupling proteins have been identified with homology to UCP1 and termed UCP2, UCP3, UCP4, and BMCP1 (*7*−*11*). Free fatty acids, liberated by noradrenergic stimulation of lipolysis, are the substrate for brown fat thermogenesis, and are also assumed to act as the cytosolic second messenger by which noradrenaline activates UCP1 (*13, 14*). However, others (*16*) exclude a role of free fatty acids as intracellular physiological activator. Evidence that fatty acids activate the integral H^+ transport property of UCP1 in BAT mitochondria has long been known (*17, 18*) and confirmed in vesicles reconstituted with the isolated protein (*19, 20*). Recently, fatty acids and nucleotides have also been discussed as physiological regulators of the H^+ conductance using white adipocytes from UCP1 transgenic mice and BAT mitochondria of UCP1-deficient mice (*21, 22*). The mechanism by which fatty acids activate UCP1 is a matter of debate. Whereas we assume that the carboxyl groups of fatty acids operate in the H^+ translocation pathway in conjunction with further resident H^+ donor/acceptor side chains of the UCP1, others regard UCP as a carrier for fatty acid anions which transport H^+ in conjunction with the free flip-flop of the undissociated fatty acids (*23*).

The introduction of a high-level expression system for UCP1 in *Saccharomyces cerevisiae* opened the door to a study of structure−function relationship using site-directed mutagenesis (*24, 25*). Thus, we were able to identify two pH sensors for nucleotide binding, E190 for both NTP and NDP and H214 only for NTP (*26*−*28*). Also, a His pair (H145, H147), which is absent in UCP2, UCP3, UCP4, and BMCP1, was identified to be essential for H^+ translocation in UCP1 (*29*).

We have now extended this study to a set of four acidic residues in UCP1 by mutagenetic neutralization. With these single mutations we identify residues important for H^+ transport and one important for Cl^- transport. The separate elimination of H^+ or Cl^- transport activity in these mutants argues against the H^+ transport mechanism by the anion fatty acid cycle and supports the postulated role of fatty acid as a prosthetic group in UCP1. Further, a residue is identified as the negative background charge, which according to our mechanism of pH control should regulate the nucleoside triphosphate binding via the previously identified H214 (*27*).

† This work was supported by a grant from the Deutsche Forschungsgemeinschaft (K1 134/36-1).

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[1] Abbreviations: wt, wild type; UCP, uncoupling protein; BAT, brown adipose tissue; FA, fatty acid; NTP, nucleoside triphosphate; NDP nucleoside diphosphate; CCCP, carbonylcyanide *m*-chlorphenylhydrazone.

10.1021/bi992448m CCC: $19.00
Published on Web 03/03/2000

MATERIALS AND METHODS

Materials. *n*-Decylpentaoxyethylene ($C_{10}E_5$) and Dowex 1-X8 (200−400 mesh) were obtained from Fluka. [^{14}C]GTP and [^{14}C]ADP were from Amersham Corp. 2′-*O*-Dansyl-GTP (2′-*O*-[5-(dimethylamino)naphthaline-1-sulfonyl]GTP) was synthesized as described by Huang and Klingenberg (*30*). The fluorescence dyes MQAE (*N*-(ethoxycarbonylmethyl)-6-methoxyquinolinium bromide) and pyranine (8-hydroxpyrene-1,3,6-trisulfonic acid, trisodium salt) were purchased from Molecular Probes.

Methods. UCP1 was expressed in the diploid yeast (*S. cerevisiae*) strain W303 transformed with vector pEMBLyE4 which contains the coding sequences for hamster brown adipose tissue wild-type UCP1 or the mutants (D27N, D27E, E167Q, D209N, D210N, and D209N + D210N). Gene expression was under the control of gal10/cyc1 promoter. For the preparation of mitochondria, yeast cells were grown at 29 °C in selective lactate medium, and expression was induced by adding 0.4% galactose. Mitochondria were isolated from protoplasts as previously described (*26*, *27*).

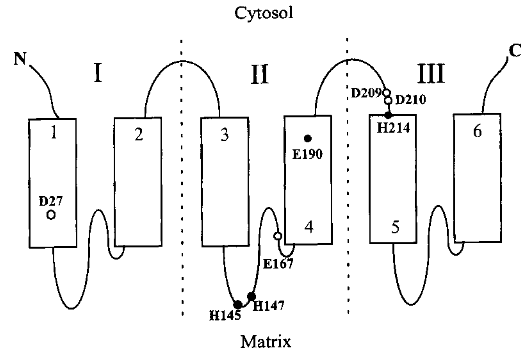
Respiration of UCP1-containing mitochondria was determined by polarography with the platinum electrode at 25 °C. For measuring respiration, mitochondria (0.1 mg/mL) were suspended in a buffer consisting of 0.65 M sorbitol, 10 mM KCL, 10 mM KH_2PO_4, 10 mM Tris, and 0.05% bovine serum albumin, pH 6.8. Substrate for respiration was 3 mM NADH. Lauric acid (100 μM) was added to induce uncoupling and GTP (500 μM) for discerning the UCP-linked activation of respiration.

H^+ transport of mitochondria was recorded with a fast-response pH electrode (Russel, Scotland) (*31*). Isolated mitochondria (1.5 mg/mL) were resuspended in 0.6 M mannitol, 100 mM KCL, 2.5 mM KH_2PO_4, and 1 mM Mops, pH 7.2, to a total volume of 300 μL. Electron transport was blocked by addition of 4 μM antimycin, and the ADP/ATP carrier was inhibited by the addition of 5 μM bongkrekate and 10 μM carboxyatractylate. Lauric acid (50 μM) was added as an activator of UCP1. The H^+ release, initiated by addition of 2 μM valinomycin, was calibrated by addition of 10 mM H_2SO_4 in steps over 20 nmol of H^+. The total H^+ release capacity was recorded after addition of 2 μM CCCP. The H^+ transport inhibition was measured in the presence of 50 μM GDP.

H^+ and Cl^- transport into reconstituted phospholipid vesicles and nucleotide binding to mitochondria, vesicles, and isolated protein follow the protocol described previously (*26, 27, 29, 32*).

RESULTS

Mutagenized Residues. In this work four different, potentially interesting acidic groups in UCP1 were mutagenized into neutral residues. Our aim was to determine the possible involvement of the carboxyl group in H^+ transport and in pH regulation of nucleotide binding. D27 is located in the putative first transmembrane helix (Figure 1) and is present uniquely in all members of the UCP family. Interestingly at the same position a lysine is found in the ADP/ATP carrier (AAC) family and a histidine in the phosphate carrier (*33*). The double adjacent aspartates D209 and D210, located in the stretch between the fourth and fifth helices at the cytosolic side (Figure 1), were selected because of their neighborhood



27 145 147 167 190 209 210 214
haUCP-1...CLA***D***I IT....QS***H***L***H***G I.....ATT***E***SFS.....TVL***E***VC:....QIL***DD***VPC***H***LLSA...
hUCP-1.....CLA***D***VIT...QS***H***L***H***G I.....ATT***E***GLT.....NCT***E***LV......ILA***DD***VPC***H***LVSA...
hUCP-2.....CI A***D***LT I...QA***R***AGGG.. .AR***EE***GFR....NCA***E***LV....LMT***DD***LPC***H***FTSA...
hUCP-3.....CFA***D***LTV....S I***H***LGP S.... AR***EE***GVR...NCA***E***VV....LLT***D***NFPC***H***FVSA...
hUCP-4.....TVA***E***LAT....EG***KRK***LE.....LA***E***GGIR...NMG***D***LT.....PL***ED***NIMT***H***GLSS...
hBMCP-1..IVA***E***FGT....QGSLFQG.....YQQ***E***GTR...VGV***E***LP....MMG***D***TILT***H***FVSS...

FIGURE 1: (A, top) Proposed membrane-spanning model of uncoupling protein (UCP1) showing the mutated residues. The open circles correspond to the mutated residues studied in this paper and the closed circles to the previously studied residues. (B, bottom) Amino acid sequences of UCP2, UCP3, UCP4, and BMCP1 with only the "homologous region" containing the studied residues in UCP1. The residues of UCP1 mutated in this study and in the previous ones are numbered. Bold and italic residues enhance the conservation and differences of the mutated residues between the UCPs.

to H214. In our previous work (*27*), it was postulated that H214 is under the electrostatic influence of the negative background group, which controls its position in the phosphate pocket of the nucleotide binding site, depending on the ionization state of H214, and thus contributes to the pH dependence of NTP binding. E167 was selected for its position in the hydrophilic matrix loop, where it possibly lines the H^+ and Cl^- translocate pathway.

Respiratory Uncoupling and H^+ Transport in UCP1-Containing Mitochondria. The uncoupling and H^+ transport properties of UCP1 and its mutants were tested in mitochondria isolated from the transformed yeast cells. Uncoupling was determined on the basis of the respiratory control. The H^+ transport rate was directly measured with mitochondria using a pH electrode. For this purpose electron transport was inhibited by antimycin, and the H^+ release was induced by K^+ uptake in the presence of valinomycin. To discern H^+ uptake by UCP1, the dependence on fatty acid and the inhibition by GDP were determined. Interestingly, the H^+ release depends on the presence not only of external K^+ but also of Cl^-. On incubating the mitochondria with 100 mM K^+−gluconate instead of 100 mM KCl, no H^+ release could be induced by valinomycin. The probable events of ion transport in this system are depicted in Figure 2A. First on incubating the mitochondria with FA and KCl, the high Cl^- gradient drives Cl^- into the mitochondria partially via UCP1. As a result a negative $\Delta\Psi$ develops inside, which also drives H^+ inside by UCP, leading to an acidification inside. On valinomycin addition, K^+ is taken up, generating a negative $\Delta\Psi$ outside, which drives out the accumulated H^+ via UCP1. Equilibrium is achieved when the internal KCl concentration has reached a level in accordance with the relationship $[H^+]_i/[H^+]_e = [K^+]_i/[K^+]_e = [Cl^-]_e/[Cl^-]_i$.

A typical recording of H^+ uptake is shown in Figure 2B with mitochondria from yeast cells with or without transfected wt UCP1. Valinomycin addition induces H^+ release,

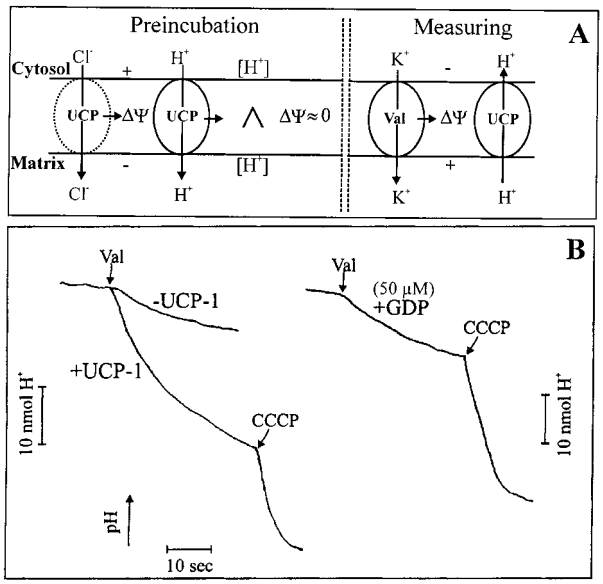


FIGURE 2: H^+ transport by UCP1 in mitochondria. (A) The scheme illustrates the ion movements involved in the measurements of H^+ transport in mitochondria. In the preincubation phase, Cl^- is taken up driven by the Cl^- gradient, and generates a negative diffusion potential inside. H^+ uptake follows, acidifying the matrix. In the measuring phase, valinomycin addition induces a positive K^+ diffusion potential inside, which drives H^+ out via UCP1. (B) Recording of electrophoretic H^+ ejection from yeast mitochondria with and without UCP1. H^+ efflux was monitored by using a pH electrode. See the Materials and Methods for the details of H^+ transport measurement.

Table 1: Transport Activities of UCP1 in Mitochondria and Reconstituted Vesicles of the wt and Mutants[a]

	mitochondria		phospholipid vesicles	
UCP-1	net H^+ transport (nmol/min mg)	GDP-sensitive portion of FA-activated respiration (%)	net H^+ transport (μmol/min mg)	net Cl^- transport (μmol/min mg)
wt	19.4	77	100	5.8
D27N	13.8	36	6.0	5.0
D27E	21	66	65	5.5
E167Q	18.8	76	90	0.2
D209N	14.6	55	76	4.5
D210N	5.5	32	24	4.5
D209N+D210N	4.8	30	21	4.2

[a] Transport activities of UCP1 in mitochondria and reconstituted phospholipid vesicles are measured as described in the Materials and Methods. Influxes are given as net transport activities obtained by subtracting the activity in the presence of 100 μM GTP or GDP. The uncoupling effect on mitochondrial respiration due to UCP is presented as the GDP-sensitive portion of fatty acid (FA)-activated respiration presented as $V_o - V_o^{GDP}/V_o \times 100$ where V_o is the respiratory rate in the presence of 100 μM FA and V_o^{GDP} is that in the presence of 100 μM GDP.

and the total capacity is recorded on further addition of CCCP. In the presence of 50 μM GDP, H^+ export is 10 times slower, whereas the total H^+ release induced by CCCP remains the same. A summary of the results on H^+ transport rate measurements with various mutants is shown in Table 1. Also the GDP-sensitive portion of respiration is given, which is measured with yeast mitochondria in the presence of 100 μM lauric acid. This is an approximate measure of the uncoupling by UCP1, but it is a rather crude indication for the H^+ conductance, since the mitochondria may be unspecifically uncoupled to different degrees according to the variability of yeast mitochondria preparation, and because of the nonproportional relation between uncoupling of respiration and H^+ conductivity. At any rate, the H^+ release rates correlate with the uncoupling degree of respiration fairly well. All mutant mitochondria exhibit GDP-sensitive respiratory uncoupling and H^+ transport activity. They are much lower in D27N, D210N, and D209N + D210N UCP1. Whereas neutralization of D27 has a stronger effect on respiration (54% less than that of the wt) than on H^+ transport (28% less than that of the wt), the results with D210N and the double mutant (D209N + D210N) are comparable, i.e., about 60−70% decrease in both respiration and H^+ transport. In D27E, E167Q, and in D209N mitochondria, on the other hand, only a 10−30% decrease was detected.

Reconstituted System. Measurements of H^+ transport in mitochondria from yeast are difficult to reproduce because of the variable intactness of mitochondria. Further they are influenced by nonspecific fatty acid effects and by the limited loading capacity for H^+ and Cl^-. Therefore, as in previous studies of UCP mutants, we isolate and reconstitute UCP into vesicles for measuring H^+ and Cl^- transport. The expressed UCP1 was purified from yeast mitochondria using the previously described protocol (*26*). Typically, a single batch of transformed yeast (20 g wet weight) yielded 30 mg of mitochondria protein with about 2.5−3.0% of expressed protein as determined from the dansyl-GTP titration and ELISA of the wt and the mutant UCP1. The yield of the isolated UCP from the hydroxylapatite column was the same in the wt and in D27E, E167Q, D209N, D210N, and D209N + D210N UCP1 but about 40% less in D27N UCP1. In the latter case, upon isolation the protein seems to be less stable. On the basis of total nucleotide binding capacity, the purity of the isolated protein was estimated to be about 70%. From this extract, UCP1 was reconstituted into egg yolk phospholipid vesicles.

As described previously, the reconstituted wild-type UCP1 catalyzed H^+ and Cl^- uptake following addition of valinomycin to proteoliposome containing internal or external K^+ medium. When 100 μM GTP or GDP was present, the rates of H^+ and Cl^- uptakes were reduced by about 85%. The average rates of H^+ and Cl^- influxes catalyzed by wt UCP1 were 120 μmol of H^+/min mg and 7.4 μmol of Cl^-/min mg of reconstituted protein. The effects of external GTP/GDP on both the H^+ and Cl^- influxes suggest right-side-out orientation of the reconstituted UCP with about 85% of the sites facing the exterior of the proteoliposomes. The remaining 10−15% H^+ transport activity represents the inverse location of the protein and the UCP-independent fatty acid−K^+−valinomycin complex diffusion (*34*). We therefore present the results (Table 1) as the net transport activity calculated by subtracting from the observed value the uptake of H^+ or Cl^- measured in the presence of 100 μM GTP or GDP.

H^+ and Cl^- Transport. D27 was mutated into a neutral residue (D27N) and into Glu (D27E). In D27N, the H^+ transport activity of UCP1 is severely affected (Table 1). In D27E, the H^+ transport activity is only slightly decreased to a rate of about 65% of that of the wt. The Cl^- transport rates of D27N and D27E are almost the same as that of the wt (Table 1) with about 80% inhibition by GTP or GDP. We conclude that the carboxyl group of D27 plays a role only in H^+ transport. Mutations of adjacent acidic residue

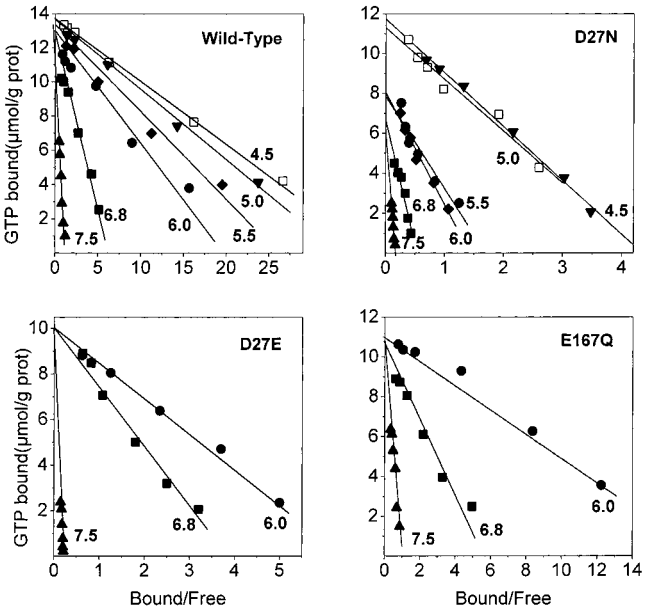


FIGURE 3: Mass action plots of [^{14}C]GTP binding to isolated UCP1 (wt, D27N, D27E, and E167Q) at different pH values. UCP1 (0.2 mg of protein/mL) was incubated in 15 mM Mops buffer and 1 mM PMSF at 0 °C for 30 min with increasing concentrations (1−16 μM) of [^{14}C]GTP. Binding was determined by the "anion exchange method" (*36*). The samples were passed through a small column (2 × 60 mm) containing 20 mg of wet Dowex 1 × 8 (Cl^- form), and the bound nucleotides were determined by scintillation counting of the eluate.

pair D209 and D210 show different effects. Neutralization of D210 decreases the H^+ transport activity about 80% and only 25% on neutralization of D209 (Table 1). With the double mutant (D209N + D210N), the H^+ uptake is about the same as with D210N (~20% of that of the wt). Both these residues have no role in Cl^- transport as indicated by the Cl^- influx rates summarized in Table 1. Therefore, among the two adjacent acidic residues at the cytosolic side of the membrane, primarily D210 seems to be essential for H^+ translocation activity of UCP1.

As a contrast case for the role of a carboxyl group in UCP, we selected E167 located at the matrix side. As presented in Table 1, neutralization of this acidic residue does not affect the H^+ transport activity of UCP1. E167Q has almost the same nucleotide-sensitive H^+ transport rate as the wt. Surprisingly, Cl^- transport activity was nearly abolished in the reconstituted vesicles of E167Q (Table 1). The Cl^- influx rate was only slightly above the baseline obtained in the presence of GTP or GDP. These results suggest a specific role of E167 for anion translocation in the UCP1 channel.

Nucleotide Binding. The concentration dependence of GTP binding yielded linear mass action plots, from which K_D and B_{max} values were obtained, which are listed in Figure 3 and Table 2. There is agreement among the affinity, the kinetics, and the pH dependency of nucleotide binding in the wt and E167Q UCP1. The K_D for GTP binding in E167Q increases from 0.61 μM at pH 6.0 to 10.3 μM at pH 7.5 comparable to the 20-fold increase in the wt. The D27 mutants show remarkably lower binding affinity for GTP in both substitutions (Asp or Glu). The K_D values are higher than that of the wt in the whole pH range studied (Table 2). The pH dependency is still retained although the decrease with pH of the binding affinity in D27N (from pH 4.5 to pH 7.5) is slightly flatter than in the wt as shown by the slope of $\Delta pK_D/\Delta pH$. The substitution with Glu in D27E produces less affinity, but the pH dependence is almost the same as in the wt.

Table 2: Dissociation Constants (K_D), Maximum Binding Capacity (B_{max}) and Rates of Binding (k_{on}) of [^{14}C]GTP to wt, E167Q, D27N, and D27E UCP1 at Different pH Values

UCP-1	pH	[^{14}C]GTP K_D (μM)[a]	[^{14}C]GTP B_{max} (μmol/g of protein)[b]	[^{14}C]GTP $k_{on} \times 10^{-3}$ (M^{-1} s^{-1})[c]
Wt	4.5	0.28	13.5	0.59
	5.0	0.36	13.2	0.47
	5.5	0.37	12.6	0.31
	6.0	0.39	12.2	0.25
	6.8	1.05	12.0	0.20
	7.5	7.90	10.2	0.03
E167Q	6.0	0.61	11.5	0.21
	6.8	1.56	11.1	0.20
	7.5	10.3	10.2	0.04
D27E	6.0	1.40	10.2	0.24
	6.8	2.50	10.0	0.15
	7.5	28.7	9.00	0.03
D27N	4.5	2.60	11.8	0.27
	5.0	2.70	11.7	0.27
	5.5	4.60	8.10	0.27
	6.0	5.60	7.90	0.22
	6.8	12.4	6.70	0.07
	7.5	35.8	6.10	0.03

[a] The K_D values were evaluated from [^{14}C]GTP titration of 200 μg/mL UCP on ice. [b] The B_{max} values were obtained from the mass action plot. [c] The rate constants were evaluated from time study of [^{14}C]GTP binding to UCP at 15 °C.

Table 2 also lists the binding rate constants for the mutants at different pH values. The rate of GTP binding to D27N is only slightly lower than that of the wt. In the glutamic acid substitution (D27E) the rate of binding is the same as that of the wt. These results argue against a role of these acidic residues in the binding of nucleotide but relate the affinity decrease of D27 substitution to the role of this residue in maintaining the conformation of the protein (see below).

The double adjacent acidic residues (D209, D210) are located within the third domain, three residues above H214, which is suggested to be located at the end of the binding pocket. It was proposed that the position of this residue (H214) is regulated by a background carboxyl group dependent on the ionization state $H214H^+$. To elucidate this role of D209 and D210, nucleoside di- and triphosphate binding to isolated mutant proteins was performed at different pH values (Figure 4). Table 3 lists the results of several binding titrations at pH 6.0, 6.8, and 7.5 for both [^{14}C]GTP and [^{14}C]ADP. At low pH (pH 6.0), neutralization of D209 causes a small increase of K_D (about 2-fold) and neutralization of D210 a stronger increase (4−fold) of the K_D for GTP binding. In contrast, at high pH (pH 7.5), these mutations slightly decrease the K_D. As a result, the strong pH dependence of GTP binding in the wt is diminished in particular by the neutralization of D210. On shifting the pH from 6.0 to 7.5, the K_D increases 8-fold in D209N and 3.4-fold in D210N as compared to a 20-fold increase in the wt. The double mutation (D209N + D210N) produces an even smaller pH dependence of GTP binding. Different from their effect on the binding of GTP, these mutations do not markedly change the K_D of ADP. At both low and high pH values (6.0 and 7.5), the K_D values are the same as in the wt, and as a result the pH dependency is retained.

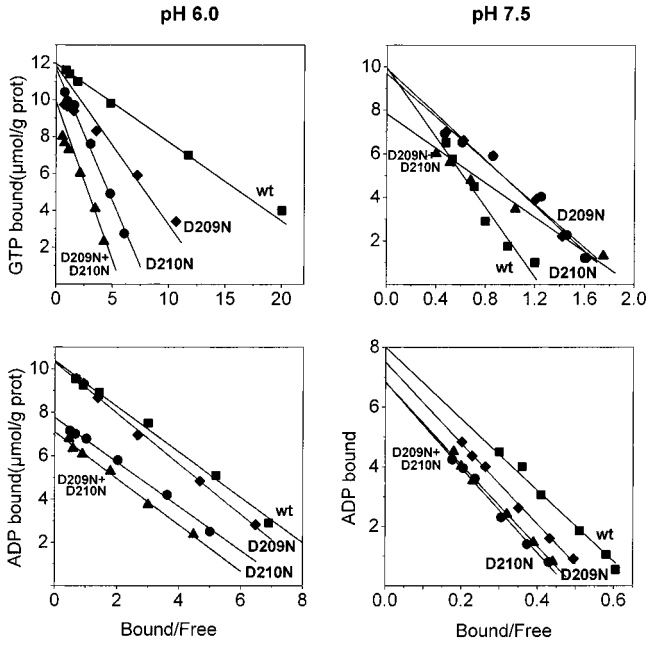


FIGURE 4: Mass action plots of [^{14}C]GTP and [^{14}C]ADP binding to isolated wt, D209N, D210N, and D209N + D210N UCP1 at pH 6.0 and 7.5. Binding measurement conditions as described in Figure 3.

Table 3: Dissociation Constants (K_D) by [^{14}C] GTP and [^{14}C] ADP and Rates of [^{14}C] GTP Binding to Wt, D209N, D210N and D209N+D210N UCP1 at Different pH

		K_D (μM)		k_{on} (M^{-1} s^{-1})
UCP-1	pH	[^{14}C]GTP	[^{14}C]ADP	[^{14}C]GTP × 10^{-3}
wt	6.0	0.39	1.05	0.25
	6.8	1.05	3.16	0.20
	7.5	7.90	13.0	0.03
D209N	6.0	0.64	1.10	0.27
	6.8	1.78	3.16	0.27
	7.5	5.20	13.5	0.07
D210N	6.0	1.49	1.02	0.22
	6.8	2.01	3.12	0.17
	7.5	5.00	14.3	0.06
D209N + D210N	6.0	1.46	1.07	0.28
	6.8	2.60	3.03	0.24
	7.5	3.49	13.8	0.09

The same decreased pH sensitivity was also shown in the rates of binding [^{14}C]GTP to isolated mutant proteins. For the D209N, D210N, and D209N + D210N mutants the time progress of binding clearly illustrates the faster binding rate at high pH, although at low pH the kinetics of binding is the same as that of the wt. The k_{on} values of D209N, D210N, and the double mutant decrease only about 3.5-fold from pH 6.0 to pH 7.5 as compared to an 8.5-fold decrease in the case of the wt. The pH dependence of the binding rate constants is plotted in Figure 5. For wt UCP, the k_{on} decreases with pH according to $\Delta pk_{on}/\Delta pH \approx 1$, whereas in the double acidic pair mutants the k_{on} changes less between pH 6.5 and pH 7.5, resulting in a slope of $\Delta pk_{on}/\Delta pH \approx 0.4$.

Stability of the Isolated and Reconstituted Mutant Protein. To check whether neutralization of these acidic residues affects the incorporation of protein into vesicles, and thus to confirm that the defects of the mutants in transport activities are not due to a misfolding of the reconstituted protein, we determined [^{14}C]GTP binding to the reconstituted proteoliposomes. On the basis of the measurements (data not shown), both for mutants and for wt UCP1, 70−80% of the protein offered was incorporated into vesicles. These results suggest that neutralization of these acidic residues does not affect the incorporation of the UCP1 in vesicles.

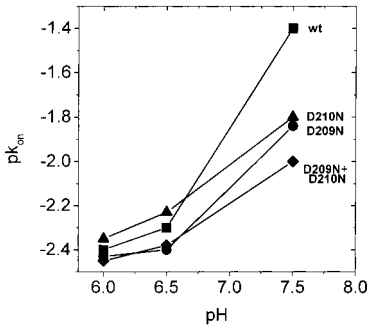


FIGURE 5: pH dependence of binding rates (k_{on}) of UCP1 (wt, D209N, D210N and D209N + D210N). pk_{on} measured by [^{14}C]GTP binding in Mes or Mops buffer (10 mM) at 15 °C as described in refs *26*, and *28*).

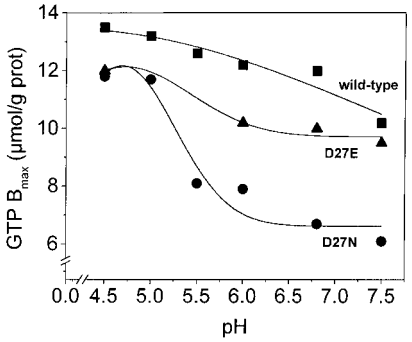


FIGURE 6: Maximum binding capacity (B_{max}) as a function of pH for isolated wild-type and D27 mutant UCP1. B_{max} values were determined from [^{14}C]GTP titrations as described in Figure 3.

The influence of mutation on the stability of the isolated protein was examined on the basis of the total [^{14}C]GTP binding capacity (B_{max}) as a function of pH. As shown in Figure 6, B_{max} of the wt UCP changes slightly from pH 4.5 to pH 7.5 but decreases dramatically in D27N from pH 5.0 to be half at pH 7.5, whereas in D27E it is almost the same as in the wt. Also, the difference in the H^+ transport activity between mitochondria and the reconstituted D27N reflects the role of this intrahelical residue in maintaining the stability of the protein. Whereas the H^+ transport activity was almost abolished in the reconstituted D27N, it was reduced by only 50% in the respiration measurements at the mitochondrial level. Most probably this residue forms an ion pair with other intrahelical positive residues in the protein and thus maintains a stable native structure. Neutralization of the other extrahelical acidic residue (E167, D209, D210) does not affect the stability of the isolated protein.

DISCUSSION

The interest for prominent carboxyl groups within the UCP structure resides in two possible roles in the function of UCP, H^+ transport and pH control of nucleotide binding. An unexpected third role reported here is facilitation of Cl^- transport. UCP1 contains two conspicuous acidic groups within the transmembrane region, in the first helix D27 and in the fourth helix E190. Interestingly both residues are conserved in the variants of UCP1, i.e., in UCP2, UCP3, UCP4, and BMCP1 and even in the UCP homology in plants

(*7*−*12*). The conservation of these residues in the UCP family despite a similarity of only 57%, 53%, 30%, and 34% to UCP1, respectively, points to the importance of these residues in basic UCP functions.

Previously, the role of the intrahelical E190 was identified by chemical modification with the highly specific Woodward Reagent K (*28*) and by mutagenesis to E190Q (*26*), as a pH sensor for nucleotide binding. E190 is visualized to form a gate for the phosphate binding moiety niche with an opposing positive group. On H^+ binding, the ion bridge is released and the gate opens. A postulated second pH sensor, which exerts an additional control on the binding of nucleoside triphosphate binding (NTP) only, was identified as H214 (*27*). Its interaction with carboxyl groups will be discussed below.

D27. The other UCP-family-specific, intrahelical residue D27 is in an equivalent position where in the ADP/ATP carrier (AAC) family an intrahelical Lys (e.g., K24 in the bovine AAC1 and K37 in the yeast AAC2) is located. The exchange of a positive against a negative residue indicates a UCP-specific critical role of D27. Notably, in both cases in this position the more hydrophilic Asp and Lys instead of Glu and Arg are inserted, which occur less frequently within transmembrane helices than Glu and Arg.

In view of these considerations, D27 was mutagenized to Asn and Glu. H^+ transport is drastically reduced on elimination of this carboxylic group, but retained on replacement with Glu. The nearly full inhibition of H^+ transport in D27N in the reconstituted system differs from a small decrease of H^+ transport and 50% decrease of uncoupled respiration in mitochondria. The discrepancy can be only partially explained by the impaired stability of isolated D27N−UCP. The binding capacity (B_{max}) of D27N is reduced at pH 5.5 by half as compared to those of the wt and D27E. Possibly D27 forms an ion bond with an intrahelical Arg such as R83 or R276 located at the same helix turn. The main factor contributing to the discrepancy is the about 20 times higher H^+ transport turnover of the reconstituted as compared to the intramitochondrial UCP. A strong decrease of activity will have much less influence on the slower H^+ transport in mitochondria.

A role of intrahelical Asp in H^+ transport has a well-characterized precedence in bacteriorhodopsin. Besides the indispensable D85 and D96 on both sides of the retinal Schiff base (*37*), at least one more additional Asp is involved in H^+ capture (*38*). In UCP the function of D27 may be to accept H^+ from fatty acids which protrude with their carboxyl group into the H^+ translocation path. D27 then delivers H^+ further into the matrix side direction. In view of the residual H^+ transport activity of D27N, D27 seems to have a role of accelerating H^+ transfer rather than being an indispensable H^+ transfer group.

The D209, D210 Pair. The double adjacent D209 and D210 are mutagenized because of their proximity to H214, which has previously been shown to be involved in the pH regulation of nucleoside triphosphate (NTP) binding (*27*). In the H214 mutants a drastic reduction of the pH dependence of NTP but not of NDP binding provides evidence that H214 matches the His postulated to be responsible for the additional pH dependence of NTP (*30*, *36*). In the ionized state, $H214H^+$ is visualized to be withdrawn from the phosphate binding pocket by a background negative residue to allow insertion of the γ-phosphate of NTP. Conversely, H214 in the neutral form is released and thus hampers NTP binding. This model gained support by replacing His with the more bulky Trp. In H214W the affinity (K_D^{-1}) is decreased about 8-fold at low pH as compared to those in the wt and H214N (*27*).

Elimination of the negative background charge should lower the binding affinity for NTP only. In fact, in D210N the affinity decreases about 4-fold at pH 6.0 but is even slightly higher at pH 7.5. The affinity for ADP and the pH dependence of NDP binding remain unaffected. These characteristics correspond to those of the postulated background negative charge, although the affinity decreases less than expected. In D210N the effect on the affinity and the pH dependence are stronger than in D209N. D210 provides obviously more directly the interacting negative background charge than D209. It is thus no surprise that the double mutant D209N + D210N has the same affinity as D210N.

The adjacent carboxyl pair provides an interesting variant to a single carboxyl group as a background charge. The pair is able to maintain on average at least one negative charge down to and even below pH 4.0. This explains the observation that no additional pK_D for the best fitting of the pK_D/pH plots of NTP binding has emerged, which should be expected from the dissociation of the background carboxyl (*36*). The binding affinity should decrease once the pH drops below pH 6.0 by abolishing the ionic bond and releasing H214 into the binding pocket. However, the carboxyl pair seems to be able to maintain the negative charge and thus to retract $H214H^+$ even at low pH. The residual binding affinity in D210N and the double mutant results from the balance of the opposing forces, i.e., the sterical hindrance from the protruding H214 and the binding energy from various contributing elements, and last but not least also from an ionic interaction of the phosphate with the $H214H^+$.

H^+ transport is also affected by the elimination of the carboxyl group in D210N. Only 20% activity is retained, but a much smaller effect is found in D209N. Interestingly, the decrease both of H^+ transport and of affinity ran parallel in these mutants. It is tempting to speculate that D210 forms a link between regulation of H^+ transport and nucleotide binding, such that by H^+ association to D210 from the cytosol the binding of NTP and the inhibition are relaxed and H^+ can be conducted into the channel.

E167. Among the acidic residues mutagenized here, E167Q is the only one where Cl^- transport is drastically reduced without affecting the H^+ transport. These results show that H^+ and Cl^- transports are independent in UCP. The reason may be either separate channels for Cl^- and H^+ transports or different requirements for the participating residues within a common pathway. The segregation of Cl^- and H^+ transports in UCP1 as evident in E167Q argues against the H^+ transport mechanism by the anion fatty acid cycle where Cl^- transport is regarded to reflect the capacity of UCP1 to transport fatty acid anions. E167 is located in what we propose as an intramembrane loop where it may line the aqueous channel (*35*). How this carboxyl is involved in Cl^- transport may be inferred from models of receptor-linked Cl^- channels. Involvement of hydroxyl groups has been invoked, which mimic water molecules and thus can assist in the dehydration of Cl^- (*39*, *40*). In UCP1, E167 may contribute to this function in addition to the unusually

high amounts of hydroxyl groups provided by the 36 Thr and Ser residues presented in hamster UCP1. Additional support for the role of E167 in Cl^- transport comes from comparison with the phosphate carrier. Mutation of E164 in the yeast phosphate carrier, which is at a position equivalent to that of E167 in UCP1, decreases the transport activity (*41*).

ACKNOWLEDGMENT

The help of Ilse Prinz in the preparation of mitochondria and that of Hendrik Schubert in the various yeast preparations are gratefully acknowledged.

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BI992448M