Accelerated Publications

H^+ Transport by Uncoupling Protein (UCP-1) Is Dependent on a Histidine Pair, Absent in UCP-2 and UCP-3†

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ABSTRACT: UCP from brown adipose tissue of hamster (now UCP-1) expressed in *Saccharomyces cerevisiae* was used to examine the role of a conspicuous histidine pair H145 and H147 which is conserved among UCP-1 from various animals. Single and double mutants were generated by converting H145 and H147 into neutral residues (H145Q and H147N). As measured by fluorescence of dansyl-GTP binding, the level of expression of the mutant UCP was the same as wild-type (wt) in the isolated mitochondria. With the isolated and reconstituted UCP, transport of H^+ and Cl^- were measured. The fatty acid dependent H^+ transport was reduced to about 10% in the single mutant H145Q and H147N and almost abolished in the double mutant, whereas Cl^- transport into these vesicles was not affected as compared to wt. The possible involvement of the His pair in nucleotide binding and its pH dependence were examined by determining the K_D and the kinetics for [^{14}C]GTP and [^{14}C]ADP binding. There were no marked changes in the affinity as well as in the binding and dissociation rates toward both these nucleotides in the mutant versus wt. Thus, the involvement in nucleotide binding can be excluded. The His pair is localized on the matrix side, probably at the entrance of the H^+ translocation channel in UCP-1. It is absent in the recently discovered UCP-2, and therefore, UCP-2 might be predicted not to be a H^+ transporter or to use a different mechanism. UCP-3 is deficient only in the equivalent H145 and thus can be predicted to still sustain a reduced H^+ transport. The data support our contention that H^+-dissociation side chains of UCP-1 are involved in H^+ transport in cooperation with fatty acid carboxyl groups.

The uncoupling protein (UCP-1)[1] of brown adipose tissue mitochondria is a H^+ transporter which short-circuits H^+ pumped by the respiratory chain and thus generates heat (*1*−*3*). H^+ transport activity requires the presence of free fatty acids and is inhibited by purine nucleotides (ATP, ADP, GTP, and GDP) (*4*, *5*). The inhibition by nucleotides is strongly pH dependent (*6*).

In a way, UCP is the simplest H^+ transporter known so far, not dependent on ATP hydrolysis or on light-induced H^+ switches. However the mechanism of H^+ transport in UCP is still unresolved. In particular the role of fatty acids in H^+ transport is a matter of controversy. Whereas we assume that fatty acids provide H^+-carrying groups within the UCP, 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 (*7*). In our model, the carboxyl groups of fatty acids operate in the H^+ translocation pathway in conjunction with further resident H^+ donor/acceptor side chains of the UCP (*8*).

UCP is a member of the mitochondrial carrier family, which also includes the most prominent one, the ADP/ATP carrier. Whereas the ADP/ATP carrier is ubiquitous in mitochondria from all cells, UCP has been thought to be confined only to mammalian brown adipose tissue. Recently however two isoforms, UCP-2 and UCP-3, have been detected also in other organs of mammalians, where they may play an important role in controlling basic metabolic rate and, thus, obesity (*9*−*14*). Heterologous expression in yeast cells indicated that also UCP-2 and UCP-3 cause uncoupling of mitochondria (*9*, *10*). It will be of greatest interest to probe the functional difference between the three isoforms of UCP and to correlate them to structural differences. Our ongoing work on site-directed mutagenesis of UCP-1 expressed in yeast is attacking this problem by examining the effects of mutation on the transport activities and its regulatory parameters. To rigorously exclude interfering components in our experiments, UCP is isolated and

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[1] Abbreviations: wt, wild-type; UCP, uncoupling protein; hUCP, human uncoupling protein; MQAE, *N*-(ethoxycarbonylmethyl)-6-methoxyquinolinium bromide; dansyl-GTP, 2′-*O*-[5-(dimethylamino)naphthaline-1-sulfonyl]-GTP; $C_{10}E_5$, *n*-decylpentaoxyethylene; CCCP, carbonylcyanide *m*-chlorphenylhydrazone; PMSF, phenylmethanesulfonylfluoride; HTS, hydroxyapatite.

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reconstituted for the functional assays.

In this communication, we identify for the first time in UCP the existence of H^+ donor/acceptor groups as the His pair H145 and H147. Mutagenesis into glutamine and arpargine blocks specifically H^+ transport. All other functions of UCP, like Cl^- transport, nucleotide binding, and pH dependence of nucleotide binding, are retained. This finding has not only important consequences for understanding the mechanism of H^+ transport in UCP, but also is of greatest interest in view of the striking absence of this His pair in UCP-2 and UCP-3, indicating that in UCP-2 and UCP-3 either no H^+ transport occurs or the transport mechanism and regulation is different from UCP-1.

MATERIALS AND METHODS

Materials. *n*-decylpentaoxyethylene ($C_{10}E_5$) and Dowex 1 × 8 (200−400 mesh) were obtained from Fluka. [^{14}C]-GTP and [^{14}C]ADP were from Amersham. 2′-*O*-Dansyl-GTP was synthesized as described by Huang and Klingenberg (*15*). The fluorescence dyes MQAE and pyranine (8-hydroxpyrene-1,3,6-trisulfonic acid, trisodium salt) were purchased from Molecular Probes.

Mutagenesis. The gene coding for UCP-1 from hamster was cloned in pEMBLyEx4 vector (*16*) under the control of the gal10/cyc1 promotor as described earlier (*17*). Histidine mutants were generated by using an oligonucleotide-directed system (USE Mutagenesis Kit, Pharmacia). The CAT codon for H145 and CAC for H147 were changed to CAG codon for glutamine and AAC for aspargine to construct H145Q and H147N. The double mutant H145Q+H147N was constructed by one oligonucleotide containing the codons as described for the single mutations. The sequence of the mutants was verified by DNA sequencing, also checking for the absence of any other mutation in the UCP-coding frame. The *Saccharomyces cerevisiae* strain W303 was transformed with plasmid containing the mutation. Yeast transformants were grown in selective lactate medium, and expression was induced by adding 0.4% galactose (17).

Isolation of Mitochondria and UCP Quantification. Mitochondria were isolated from yeast by differential centrifugation, following a procedure previously described (*17*). Mitochondria were suspended in a solution containing 0.6 M mannitol/20 mM Tris, pH 7.4, containing 0.5 mM EDTA, 0.1 mM EGTA, and 1 mM PMSF. Quantification of UCP incorporated in yeast mitochondria was performed by fluorescence titration with dansyl-GTP. To remove endogenous residual bound nucleotide, mitochondria were shaken with Dowex as mentioned previously (*18*).

Isolation, Purification, and Reconstitution of UCP. UCP was isolated from yeast mitochondria using Triton X-100 as detergent according to the protocol that have been described for hamster brown adipose tissue mitochondria (*19*). The copurification of porin was largely avoided by reducing the ratio of Triton X-100 to protein to 1.2 (mg/mg). ADP/ATP carrier was largely excluded by increasing the volume of HTS four times more than that used for UCP extract from hamster mitochondria. Protein concentration was determined according to Lowry et al. (*22*) using bovine serum albumin as a standard. Purified UCP was reconstituted into phospholipid vesicle following a protocol previously described (*20*) with modification as in Echtay et al. (*17*). Briefly, UCP was suspended with egg yolk phosphatidylcholine (phospholipid:protein = 500:1 by mass), an internal medium of 100 mM K^+ phosphate, pH 7.6, for measurement of H^+ transport or 100 mM Na^+ phosphate, pH 6.8, of Cl^- transport in addition to 0.2 mM EDTA and 1 mM PMSF, and $C_{10}E_5$ (detergent:phospholipid = 1.4 by mass). Vesicle was formed by slow removal of the detergent with Bio-Beads SM-2 at 4 °C. The external solute was removed by passing the vesicles over G-75 column. Vesicles for Cl^- transport measurement were loaded by MQAE through diffusion for 17 h in dark at 4 °C.

Fluorescent Measurement for H^+ and Cl^- Transport. The applied methods for transport measurements resemble those described previously (*17*). H^+ uptake activity was measured on MPF-44A fluorescence spectrophotometer using pyranine fluorescence at λ_{exc} = 467 nm and λ_{em} = 510 nm in a standard medium containing 280 mM sucrose, 0.5 mM Hepes, 0.2 mM EDTA, 1 μM pyranine, pH 6.9, and 125 μM lauric acid. Valinomycin (2.5 μM) was added to initiate the K^+ gradient driven H^+ uptake. Cl^- transport was measured by fluorescence of MQAE-loaded proteoliposome at λ_{exc} = 355 nm and λ_{em} = 460 nm in a medium containing 4 mM Na^+ phosphate, pH 6.8, and 155 mM KCl. Cl^- influx was initiated by the addition of valinomycin (2 μM) and the rate (J_{CL}) was determined using a two point calibration method as described by Verkman et al. (*23*) by the following equation:

$$J_{CL} = [(F_i - F_S)(K_{Cl} + 1/[Cl]_S)]^{-1} [dF(0)/dt] \quad (1)$$

where K_{Cl}, Stern−Volmer constant, is equal to 0.15 mM^{-1}.

Nucleotide Binding Measurements. [^{14}C]GTP and [^{14}C]-ADP binding titration followed in principle the published procedure using Dowex for removal of free radiolabled nucleotide (*6*). The binding rate of [^{14}C]GTP was measured with an automated rapid mixing and separating sampling machine developed in our laboratory as described previously (*17*). Nucleotide fluorescence derivative (dansyl-GTP) was applied for GTP dissociation rate determination. The dissociation is followed by the fluorescence decrease on addition of large excess ATP after fluorescence equilibrium of dansyl-GTP binding to UCP at λ_{exc} = 350 nm and λ_{em} = 525 nm. The buffer used was 10 mM Mops or Mes at 15 °C.

RESULTS

Characterization of UCP Histidine Mutants Expressed in S. cerevisiae. Out of the four histidines in hamster UCP-1, H145, H147, and H214 are conserved in all UCP-1 from other species known so far, whereas H9 is found only in a few species. The conservation of the striking (H145-L146-H147) motif prompted us early to speculate that these histidines may play an important functional role. Two possibilities were considered, a role in the pH control of nucleoside triphosphate binding or a role in H^+ transport. The strong pH dependence of nucleotide binding and concomitant inhibition of H^+ transport had been related to the protonation of a carboxyl group for the di- and triphosphate and additionally of a histidine group exclusively for the triphosphates (*6*, *15*). Recently, the intrahelical E190 has been identified as a pH sensor regulating nucleoside di- and triphosphate binding (*17*, *21*).

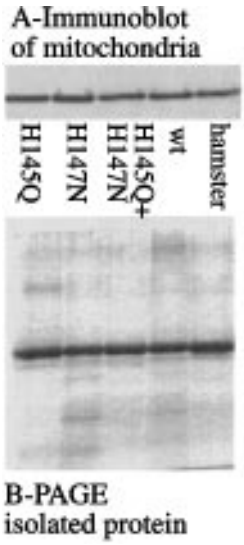

FIGURE 1: Analysis of expression and purification of wild-type and histidine mutants UCP. (A) immunoblot of yeast mitochondria protein (30 μg) using antisera against UCP. (B) SDS−PAGE, 12.5% polyacrylamide, of isolated UCP (10 μg).

Using the expression of hamster UCP-1 in *S. cerevisae*, H145 and H147 were converted into neutral residues by site-directed mutagenesis. Three variants were generated, H145Q, H147N, and H145Q+H147N. Figure 1A documents by immunoblots of mitochondria isolated from *S. cerevisiae* that UCP is expressed at approximately similar levels in mitochondria from wt and three mutant strains. Quantification of the UCP content in mitochondria was performed by titration with dansyl-GTP (not shown) and found to be similar for wt and all three mutant UCP at about 2.5% of the total protein content of mitochondria. For the function studies, UCP was isolated and purified on hydroxyapatite. According to Coomassie blue stained gel (Figure 1B), both wt and mutant UCP are quite pure (estimated to about 70% based on nucleotide binding) and isolated in about equal amounts with a yield of 1.2−1.5% of mitochondrial protein. The measurement of nucleotide binding served two purposes, to quantify the amount of UCP expressed in mitochondria and isolated therefrom and to examine whether those histidines are involved in the binding and/or specifically in the postulated pH regulation of the nucleoside triphosphate binding.

H^+ and Cl^- Transport. According to our model of H^+ transport by UCP, free fatty acids provide a carboxyl group as H^+ donor/acceptor in conjunction with resident H^+-transferring groups. In view of this potential role of the histidines, UCP was isolated and reconstituted into phospholiposomes for transport measurements using pyranine as pH indicator. The vesicles were loaded with high concentrations of K^+ phosphate as H^+ accepting buffer. As activator of H^+ transport, 125 μM lauric acid was added. Recordings of H^+ uptake into UCP proteoliposomes are shown in Figure 2A. Addition of valinomycin creates a K^+ diffusion potential positive outside. At this point in wt UCP proteoliposomes, a rapid H^+ uptake is recorded by the fluorescence increase. The H^+ uptake rate is inhibited to 85% by prior addition of 100 μM GTP. The same inhibition is observed with GDP (data not shown). The GTP-resistant H^+ uptake can be attributed to reversed incorporated UCP which cannot be accessed by GTP and to unspecific H^+ transport pathways,

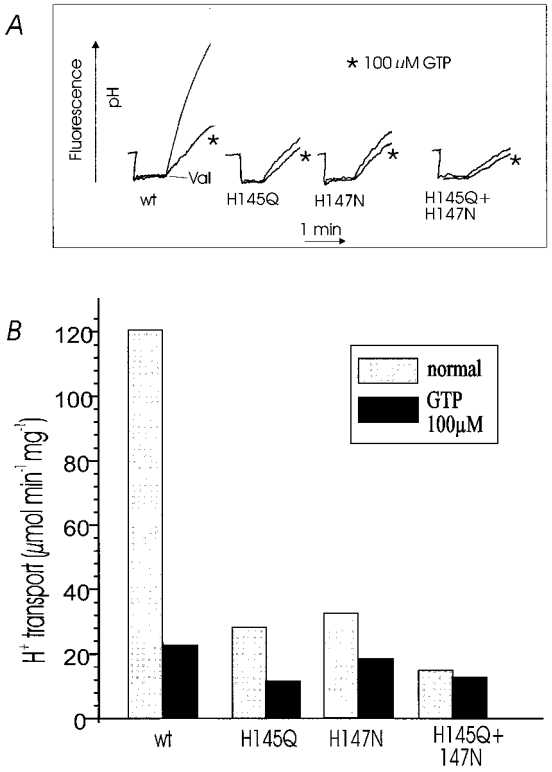

FIGURE 2: Proton influx into phospholipid vesicles reconstituted with purified wild-type and histidine mutants UCP-1 at pH 6.9. (A) recordings of H^+ uptake in reconstituted UCP liposomes. (B) evaluated H^+ transport rates. H^+ influx was measured as the change in external pH monitored by pyranine fluorescence at $\lambda_{exc} = 467$ nm and $\lambda_{em} = 510$ nm. A 50 μL portion of vesicles was added to 0.5 mM Hepes buffer, pH 7.5, containing 1 μM pyranine, 0.5 mM EDTA, and 280 mM sucrose to a final volume of 330 μL at 10 °C. Valinomycin of final concentration 2.5 μM was added to generate membrane potential in the presence of 125 μM laurate. H_2SO_4 was added in steps of 20 nmol H^+ to adjust the pH to 6.9. The uncoupling CCCP (1 μM) was used to determine the capacity of H^+ conductance across the vesicles.

such as by K^+-valinomycin-fatty acid anion complexes (*20*). In proteoliposomes containing H145Q UCP or H147N UCP at the same level as wt UCP liposomes, valinomycin induces a much slower H^+ uptake. In the presence of GTP the uptake is inhibited to the same residual rate as wt UCP. The H^+ uptake rates are similarly low with both H145Q and H147N UCP. With the double mutant H145Q+H147N UCP, the H^+ uptake rate is still lower and GTP does not further decrease the H^+ transport. We may conclude that in H145Q+H147N UCP, H^+ transport capability is completely abolished. The H^+ transport rates evaluated from these types of measurements are summarized in Figure 2B. Taking only the GTP-sensitive H^+ transport, H145Q and also H147N have lost about 85% of the wt H^+ transport capacity. In the double mutant H145Q+H147N UCP, virtually no H^+ transport is possible.

In view of the fact that UCP also has the ability to transport Cl^- (*24*), we were able to ask the question whether H145 and H147 are required only for H^+ transport and not for the ability to conduct small anions. Cl^- uptake into UCP proteoliposomes was measured using the anion-sensitive fluorescent dye MQAE. A K^+ diffusion potential was generated by valinomycin-induced K^+ influx creating a membrane potential positive inside. No fatty acids are added since they do not influence the ability of UCP to conduct

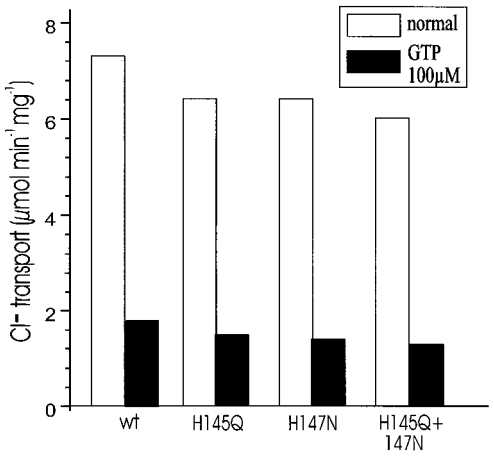

FIGURE 3: Chloride transport into phospholipid vesicles reconstituted with purified wild-type or histidine mutants UCP-1 at pH 6.8. Cl^- influx was monitored by fluorescence of MQAE loaded into the vesicle at λ_{exc} = 355 nm and λ_{em} = 460 nm. A 50 μL portion of vesicles was suspended in 4 mM sodium phosphate buffer containing 155 mM KCl to a final volume of 385 μL at 10 °C. The Cl^- influx rate was monitored after addition of 2 μM valinomycin. Tributyltin acetate (40 μM) was added to equilibrate internal and external chloride. The influx rate in units of micromoles per minute milligram of protein were calculated from the fluorescence data by using eq 1.

Cl^- or other anions. The measurements of Cl^- transport with reconstituted wt and mutant UCP are summarized in Figure 3. In wt UCP, the transport of Cl^- is about 15 times slower than that of H^+ under these conditions. There is no marked decrease of Cl^- transport activity in the three mutants as compared to wild-type. Cl^- transport is inhibited by GTP and GDP to the same extent in the wt and the mutants. These results show that these mutants still retain the anion transport activity of UCP and the capability to inhibit the conductance by nucleotides. It must be concluded that the histidine pair H145 and H147 are specifically involved in H^+ transport but not in the more basic transport activity of small anions.

Nucleotide Binding. The concentration dependence of GTP binding was studied at pH 6.0, 6.8, and pH 7.5. No marked binding differences between the wt and the three histidine mutants were observed. The binding capacity is nearly identical from pH 6.0 to 7.5. The binding affinity as deduced from the linear slope in the mass action plots decreases strongly going from pH 6.0 to 7.5 (data not shown). The decrease is similar between the four different UCP, i.e., the binding affinity decreases with the pH to the same degree in the mutant as in the wild-type. The affinity of ADP to the His mutant UCP was pH sensitive in the same way as that to wild-type. These data are summarized in Table 1.

As previously shown, the binding rate of [^{14}C]GTP to isolated UCP from yeast can be measured by an automated mixing, sampling, and separation apparatus (*17*). In order to exclude further the involvement of H145 and H147 in the pH regulation of nucleoside triphosphate, a kinetic study of GTP binding and dissociation to histidine mutants was performed. The rate constants (k_{on}) were evaluated according to the second-order reaction of [^{14}C]GTP binding and the k_{off} values were measured using the fluorescence nucleotide derivative (dansyl-GTP) binding. No marked rate constant differences between the wt and the histidine mutants were observed. The pH dependency of the binding and dissociation rate constants is plotted in Figure 4. As measured for

Table 1: Dissociation Constant (K_D) of Wild-Type and Histidine Mutants UCP-1 by [^{14}C]GTP and [^{14}C]ADP at Different pH

UCP	pH	[^{14}C] GTP[a] K_D (μM)	[^{14}C] ADP[a] K_D (μM)
wild	6.0	0.5	0.65
	6.8	1.6	1.95
	7.5	7.9	18.2
H145Q	6.0	0.7	0.60
	6.8	1.4	1.72
	7.5	5.3	19.5
H147N	6.0	1.1	0.90
	6.8	1.0	2.19
	7.5	5.6	19.6
H145Q+H147N	6.0	0.62	0.66
	6.8	1.0	2.17
	7.5	6.0	16.0

[a] The K_D values were evaluated from [^{14}C]GTP and [^{14}C]ADP titration of 200 μg/mL UCP on ice.

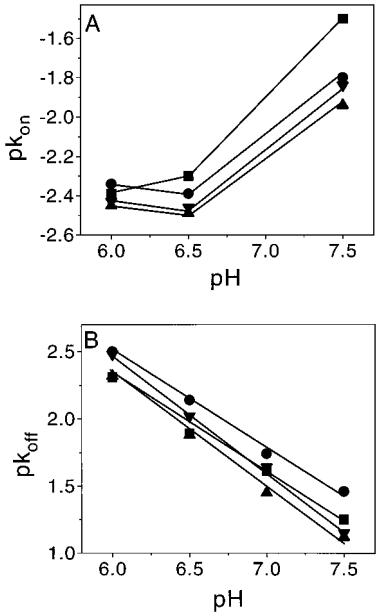

FIGURE 4: pH dependence of binding and dissociation rates. (A) pk_{on} measured by [^{14}C]GTP binding and (B) pk_{off} measured by dansyl-GTP binding. Measurements in Mes or Mops buffer (10 mM) at 15 °C as described in Materials and Methods. (■) wild-type, (●) H145Q, (▲) H147N, and (▼) H145Q+H147N UCP.

[^{14}C]GTP, the binding rate decreases with pH showing a slope of ~1 in the pk_{on}/pH plot, and the dissociation rate of dansyl-GTP increases with a $\Delta pk_{off}/\Delta pH \approx -1$ of both wt and histidine mutants.

DISCUSSION

The thermogenesis of UCP is thought to be intimately linked to the ability to conduct H^+. UCP is also able to transport small anions, in particular Cl^-, for as yet unknown physiological reasons. H^+ transport requires, as cofactor, free fatty acids, which serve at the same time as a major fuel for the brown adipose tissue mitochondria. Understanding the H^+ transport mechanism in UCP poses a major challenge, although UCP can be considered to be the simplest H^+ transporter known. Some groups consider that UCP is not an actual H^+ transporter but facilitates the translocation of fatty acid anions which freely recycle through the membrane in the protonated state (*7*, *25*). In contrast, we have maintained that UCP contains H^+-conducting groups and that fatty acids are a cofactor providing an additional

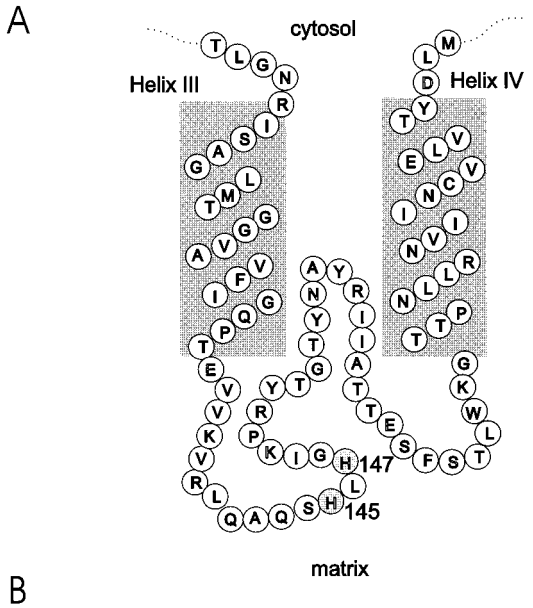

FIGURE 5: (A) Scheme illustrating the putative positions of the mutated His pair H145Q and H147N in UCP-1. (B) Comparison of homologous amino acid sequence sections among the hamster UCP-1, hUCP-1, hUCP-2, and hUCP-3, respectively.

but necessary H^+-conducting group, which operates in conjunction with the resident groups (*8*). H^+-conducting side chains in UCP could be either carboxyl or imidazol groups. With the reported results, we demonstrate the existence of two H^+-conducting groups in UCP, H145, and H147. Each H145 and H147 alone is still able to sustain 10% of wt H^+ transport activity, but their combined elimination completely abolishes H^+ transport. In other terms, each histidine alone overcomes the H^+ conducting barrier partially, and only the combined action allows high H^+ conductivity. The suppression of H^+ but not of Cl^- transport by these mutations argues against the H^+ transport mechanism by the anion fatty acid cycle (*7*).

This role of His in H^+ transfer is surprising in view of its position according to the conventional folding model at the matrix side of the UCP. The His pair can be visualized to be localized at the matrix end of the H^+ translocating pathway (Figure 5A). Within the transmembrane helices, two carboxyl groups, E190 and D27, could be further candidates of H^+ translocating groups. However, E190 has recently been shown by site-directed mutagenesis not to participate in H^+ transport but instead being the pH regulator of nucleotide binding (*17*). D27, highly conserved in all UCP, remains a candidate to work in conjunction with the two histidines and the fatty acids as the H^+ conductor. What could be the role of His versus carboxyl groups in the H^+ transfer by UCP? His has a p$K \approx 6.5$ in hydrophilic environment whereas the pK of Glu or Asp carboxyl groups in proteins can range from p$K \approx 2.5$ to 8.0 depending on the environment (*26*). In general, the pK of carboxyl groups is much lower than of His, and thus, H145 and H147 could generate a H^+-accepting gradient in UCP toward the matrix side, in line with the uncoupling function. It should be noted that in UCP the pK gradient across the membrane should be different from the well-defined H^+ pumping channels as in bacteriorhodopsin or photosynthetic reaction center (*27*, *28*), since in UCP, H^+ travels downhill the membrane potential. The exposed position of H145 and H147 also suggests involvement in pH regulation of H^+ transport as observed in the pH dependence of H^+ transport (unpublished observation).

The involvement of His in H^+ transport is not without precedent in other proteins. The mitochondrial phosphate carrier facilitating phosphate-H^+ cotransport is also a member of the mitochondrial carrier family (*29*). The intrahelical H32, in the bovine phosphate carrier, has been shown to be essential for the phosphate-H^+ cotransport (*30*), however, a separate role in H^+ transport has not yet been proven. In the lac permease of *Escherichia coli*, H322 is essential for the H^+ cotransport. Its mutation (H322N) still tolerates galactoside transport without accompanying H^+ (*31*). Further, in the intestinal H^+-peptide cotransporters, His groups have been identified to be involved in transport activity, although no segregation of H^+ and peptide transport was achieved (*32*). The essential histidine H57 in pep T1 and H87 in pepT2 are located near the extracellular end of the second transmembrane helix. Histidines have been shown to play a role in the activity of the human placental Na^+-H^+ exchanger (*33*). A cluster of His residues serves as a pH sensor in the nonerythroid anion exchanger AE2 (*34*).

It is highly intriguing that the histidine pair H145 and H147 does not occur in the recently discovered UCP-2 and UCP-3 (Figure 5B). In particular, UCP-3 is thought to be the major thermogenic component in skeletal muscle and seems to play a major role in obesity. Whereas in UCP-2 both histidines are missing and H145 is in a homologous position replaced by arginine, in UCP-3 only the homology to H145 is retained. Extrapolating the results from UCP-1, one might predict that UCP-2 has no H^+ transport and UCP-3 a weak H^+ transport. Another suggestion might argue that fatty acids intervene in a somewhat different manner in UCP-2 and UCP-3 by replacing these H^+ translocating groups. Finally, it can be envisaged that, if there is H^+ transport in UCP-2 and UCP-3, it might be regulated by the matrix pH in a different manner as in UCP-1.

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