Mutagenesis of the Uncoupling Protein of Brown Adipose Tissue. Neutralization of E190 Largely Abolishes pH Control of Nucleotide Binding†

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ABSTRACT: For expression in *Saccharomyces cerevisiae* the cDNA of the uncoupling protein (UCP) of brown adipose tissue from hamster has been isolated and used to transform yeast cells. Optimized expression conditions yielded 2% of mitochondrial protein as UCP. UCP was isolated, avoiding copurification of ADP/ATP carrier and porin. Intrahelical E190, previously suggested to be the pH sensor for nucleotide binding, was neutralized to glutamine by mutagenesis. In binding titrations with [^{14}C]-guanosine 5′-triphosphate (GTP) and with fluorescent dansyl-GTP, near equal binding capacity for GTP was measured in wild-type (wt) and E190Q. The K_D for GTP binding to UCP from yeast has the same strong pH dependence as the original UCP from hamster. With both [^{14}C]GTP and dansyl-GTP, the K_D in wt increased 16−19-fold from pH 6.0 to 7.5, while in E190Q this increase was only 2.5−2.9-fold. As a result, at pH 7.5, both [^{14}C]GTP and dansyl-GTP bind 6-fold tighter to E190Q than to wt. The binding rate of GTP decreased 10-fold from pH 6.0 to 7.5 in wt and only 4-fold in E190Q. Woodward reagent K (WRK) known to interact specifically with E190 [Winkler, E., Wachter, E., and Klingenberg, M. (1997) *Biochemistry 36*, 148−155] abolished [^{14}C]GTP and dansyl-GTP binding to wt UCP, whereas binding to E190Q was fully resistant to WRK. H^+ and Cl^- transport activity in reconstituted vesicles were the same with wt and E190Q. At pH 7.5, 5 μM GTP is unable to inhibit H^+ and Cl^- transport in wt but inhibits in E190Q to maximum level. The different sensitivity toward GTP versus GDP found in wt is absent in E190Q. Thus, the mutation E190Q results in the predicted gain of function in binding and proves the role of the intrahelical E190 as a pH sensor for nucleotide binding but excludes a role in transport.

The uncoupling protein (UCP)[1] of brown adipose tissue mitochondria is the key element for the thermogenesis of this tissue. It short circuits H^+ pumped out of the inner membrane by the respiratory chain (*1*, *2*). The H^+ transport is tightly controlled by various factors, such as purine nucleotides as inhibitors (*1*) and fatty acids as activators (*3*). The nucleotide binding is strongly influenced by the pH (*4*). The affinity decreases with the pH, particularly strong for the nucleoside triphosphates (*5*, *6*). The detailed analysis of this pH dependence led to the conclusion that three H^+ dissociating groups are involved, i.e., H^+ dissociation at the terminal phosphate of the nucleotides with a p*K* around 6.7 due to the preferred binding of the fully dissociated nucleotides, a carboxyl group in UCP at the binding center with a p*K* around 4, which has to be protonated for binding, and exclusively for the nucleotide triphosphates an additional carboxyl or histidine group with a p*K* 7, which also has to be protonated for binding. This intricate pH control system can strongly modulate the inhibitory effect of nucleotides of UCP.

On the basis of these results, it was proposed that a regulatory carboxyl group forms an ion pair in the dissociated form at the entrance to the phosphate moiety binding cleft (*5*, *6*). On protonization, the binding site opens and the positive residue becomes available for the phosphate moiety binding. The binding of the nucleotides was found to occur in a two-stage mode, first a rapid and loose binding and a subsequent slow transition into a tight binding (*6*, *7*). In the first stage, the nucleotide UCP complex is still active in H^+ transport and then is inhibited by the slow conformational change into the tight state as was shown by dansyl nucleotide binding studies (*7*).

Recently, the regulatory carboxyl group competent for binding of both the di- and triphosphates was tentatively identified in UCP, using Woodward reagent K (WRK) (*8*). Treatment with this reagent inhibited nucleotide binding but not H^+ transport. Highly selective and with great affinity WRK was found to modify glutamate E190 localized in the fourth transmembrane helix.

With these findings together with our model of pH control, we would predict that the removal of the carboxyl group of E190 produces UCP with no or decreased pH control of nucleotide binding. Mutagenesis of E190 should result in increased affinity and thus a gain of function. Here we report on the mutagenesis of UCP expressed in yeast converting glutamic acid 190 into glutamine. With this mutant, we are able to demonstrate the role of E190 in the pH regulation of

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[1] Abbreviations: wt, wild-type; UCP, uncoupling protein; WRK, Woodward reagent K; GTP, guanosine 5′-triphosphate; MQAE, *N*-(ethoxycarbonylmethyl)-6-methoxyquinolinium bromide; dansyl-GTP, 2′-*O*-[5-(dimethylamino)naphthalene-1-sulfonyl]-GTP; $C_{10}E_5$, *n*-decylpentaoxyethylene; CAT, carboxylatractylate; CCCP, carbonylcyanide *m*-chlorphenylhydrazone.

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the nucleotide binding and the inhibition of H^+ and Cl^- transport and thus also to vindicate the predicted model.

MATERIALS AND METHODS

Materials. Hydroxylapatite was prepared as described in Bernardi (*9*). Egg yolk phospholipid was isolated from fresh eggs and purified with Alumina B Super I from ICN Biomedicals as described previously (*10*). The detergent *n*-decylpentaoxyethylene ($C_{10}E_5$) and Dowex 1 × 8 (200−400 mesh) were obtained from Fluka, and [^{14}C]GTP was from Amersham. 2′-*O*-Dansyl GTP was synthesized as described by Huang and Klingenberg (*6*). The fluorescence dyes MQAE and pyranine (8-hydroxpyrene-1,3,6-trisulfonic acid, trisodium salt) were purchased from Molecular Probes. Restriction endonucleases and T4-DNA-ligase were obtained from Boehringer Mannheim or New England Biolabs and used as reommended by the supplier.

Isolation of cDNA From Hamster Brown Adipose Tissue. DNA manipulation, transformation of *Escherichia coli* cells (Xl1-blue) and cloning were performed as described in Sambrock et al. (*11*). PolyA-rich mRNA was prepared from brown adipose tissue of cold adapted hamster by Oligotex Direct mRNA Mini Kit (Quiagen). An aliquot of this mRNA was reverse transcribed using Reverse Transcriptase RNase H^- (Stratagene). Two primers, CAGGTC*GAGCTC*ATAATGGTGAATCCAACAACTTC for upstream and TACGCC*GGGATCCC*TATGTGGTACAATCCAC for downstream end, which are complementary to the coding sequence of UCP-cDNA, were used to perform PCR. For increasing the level of expression, the three bases before the start codon were chosen as ATA to conform the yeast consensus sequence. Primers contained unique restriction sites for *Sac*I and *Bam*HI, respectively. This allows cloning of the 925 bp DNA fragment resulting from PCR amplification in pGEM3Z plasmid (Promega). The cloned UCP coding sequence was completely sequenced by dideoxy-chain-termination mixture using a Thermo Sequenase Kit (Amersham Life Science) to check that the sequence had expected properties.

Expression in S. Cerevisiae. For the expression of UCP in *S. cerevisiae*, the coding sequence of UCP was inserted between *Sac*I and *Bam*HI restiction site of shuttle vector pEMBLyex4 (*12*) under control of the gal10-cyc1b promoter.

Yeast cells (W303D MATa Ade 2−1; His 3−11,15, Leu 2,112; Trp 1, Ura 3−52, can 1-100) were transformed by lithium acetate method (*13*). Clones containing the expression vector were selected for uracil autotrophy on plates with minimal medium (0.67% yeast nitrogen base, 2% glucose, all amino acids and adenine at 40 mg/L each, and 2% bacto-Agar).

For the expression of UCP, yeast transformants were grown in selective lactate medium (0.67% yeast nitrogen base, 2% lactic acid, 0.05% glucose, all amino acids and adenine at 40 mg/L each, pH 5.5 with KOH). Galactose was added to a final concentration of 0.4% 6−8 h before harvesting at absorbence of 3.5.

Mutagenesis. The GAG codon for glutamic acid 190 was changed to CAG codon for glutamine to construct E190Q by using an oligonucleotide-directed system (U.S.E. Mutagenesis Kit, Pharmacia) with oligonucleotide CATCAACTGTGTACAGCTGGTAACATACGAC. The sequence of the mutant was verified by DNA sequencing.

Isolation of Mitochondria. Mitochondria were prepared with some modifications following a procedure described by Gawaz et al. (*14*). Protoplasts were prepared by enzymatic digestion of cell wall with a mixture of 0.3 mg of novozym (Novo Nordisk) and 1.0 mg of zymolyase (Seikagaku Corporation)/g of wet cells in 1.2 M sorbitol, 20 mM KH_2PO_4, pH 7.2. After removing the enzymes by washing 2 times with 1.2 M sorbitol, protoplasts were homogenized and mitochondria were then isolated by differential centrifugation at 4 °C in 0.6 M mannitol/Tris 20 mM, pH 7.4, containing 0.1 % BSA, 0.5 mM EDTA, 0.1 mM EGTA, and 1mM PMSF. The final mitochondrial pellet was resuspended in the same buffer without BSA.

Isolation and Purification. Wild-type and mutant UCP were isolated from yeast mitochondria basically as described earlier for the isolation of brown adipose tissue mitochondrial UCP with some modifications (*15*). A contamination by porin was noted in the Triton X-100 extracts after hydroxyapatite pass through. The copurification of porin was largely avoided by reducing the ratio of Triton to protein to 1.2 (mg/mg). Mitochondria were solubilized at a protein concentration of 15 mg/mL for 45 min at 0 °C with Triton X-100 at a ratio of 1.2 mg of detergent:mg of protein in the presence of 20 mM Na_2SO_4, 0.2 mM EDTA, 1 mM PMSF, and 20 mM Mops, pH 6.7. The suspension was centrifuged at 100000*g* for 30 min at 4 °C. The supernatant was purified on a hydroxyapatite column (5 mL for 15 mg of mitochondrial protein) and eluted by 20 mM Na_2SO_4, 0.2 mM EDTA, and 5 mM Mops, pH 6.7, at room temperature. The UCP-containing fraction was collected and concentrated by pressure dialysis to 2 mg/mL, with a yield of about 1.2−1.5% of mitochondrial protein.

Reconstitution. The reconstitution of UCP in proteoliposome followed essentially the procedure described previously (*16*). In a typical reconstitution experiment for H^+ influx measurements, egg yolk phospholipid was emulsified and diluted by loading buffer consisting of 100 mM potassium phosphate, 0.2 mM EDTA, 1 mM PMSF, pH 7.6, and $C_{10}E_5$ at 0 °C to a final detergent:phospholipid ratio of 1.4 (by mass). The addition of isolated UCP at a phospholipid:protein ratio (500:1 by mass) resulted in concentration of phospholipid at 17 mg/mL and protein at 0.033 mg/mL. The detergent was stepwise removed by 360 mg of Bio-Beads SM-2 in 30 min intervals with gentle shaking overnight at 4 °C. For the removal of external solutes from proteoliposomes, the vesicles were passed over Sephadex G-75 (column 16 mL/mL of vesicle) equilibrated with 0.5 mM Hepps, 0.2 mM EDTA, pH 8.0, and 0.28 M sucrose to maintain external osmolarity equal to that of the internal salt concentration.

For Cl^- transport measurements, isolated UCP was reconstituted as described above with loading buffer containing 0.2 mM EDTA, 1 mM PMSF, and 100 mM sodium phosphate, pH 6.2. Detergent removal and vesicle formation were achieved by step wise addition of 500 mg Bio-Beads/14 mg of detergent for 5 h with gentle shaking at 4 °C. Vesicles were loaded with MQAE through diffusion by adding 3 mM MQAE to vesicles for about 17 h in dark at 4 °C. External MQAE and solutes were removed by Sephadex column equilibrated with 50 mM sucrose, 0.2 mM EDTA, 0.5 mM Pipes and 0.5 mM Hepes, pH 7.0. Internal volume of proteoliposome was determined after gel filtration with Sephadex G-75 based on the measurements of internal

phosphate concentration (*17*), and it was about 2 μL/mg of phospholipid.

Dansyl-GTP Binding Measurement. Fluorescence was recorded on MPF-44A fluorescence spectrophotometer (Perkin-Elmer). Titration of UCP (wild-type and mutant) with dansyl-GTP was performed in a 5 × 5 mm cuvette (300 μL) at λ_{exc} = 360 nm and λ_{em} = 515 nm. The concentration of dansyl-GTP was increased until UCP (35 μg/mL) was nearly saturated. The fluorescence was corrected for dilution (<6%). The buffer used was 20 mM Hepes, pH 7.5, or 20 mM Mes, pH 6.5. The specific fluorescence (ΔF) due to binding at the nucleotide binding site of the UCP was obtained by subtracting from the total fluorescence the residual fluorescence measured after addition of 1.5 mM ATP.

Quantification of UCP in Mitochondria. UCP was quantified by dansyl-GTP binding to isolated mitochondria (*18*). To unmask UCP from residual endogenous bound ATP, mitochondria at a concentration of 5 mg of protein/mL in incubation buffer containing 250 mM sucrose, 1 mM EDTA, 1 mM PMSF, and 20 mM Hepes, pH 8.0, were shaken with Dowex (120 mg/mg of protein) at room temperature for 1 h. For the fluorescence measurements, 300 μL of mitochondria was filled into a cuvette (5 × 5 mm) at 1 mg/mL in incubation buffer, pH 6.8, containing 10 μM CAT. Dansyl-GTP addition and calculation of ΔF were done as mentioned before.

For standardizing the estimation of UCP by the fluorescence titration, a known amount of UCP from hamster brown adipose tissue was added to yeast mitochondria containing no expressed UCP.

[^{14}C]GTP Binding Measurement. The measurement of [^{14}C]GTP binding followed in principle the published procedure using Dowex for removal of free [^{14}C]GTP (*5*). UCP (200 μg/mL) was added to a buffer containing 15 mM Mops and 1 mM PMSF. To this, [^{14}C]GTP in concentration from 1−30 μM with specific activity of 8.5 dpm/pmol was added. After 30 min incubation at 0 °C, 50 μL of the sample was applied to a column (2 × 60 mm) of 20 mg of Dowex 1 × 8 (Cl^- form) and washed twice with 100 μL H_2O. In the eluant, the bound [^{14}C]GTP was determined by scintillation counting.

For the measurement of GTP binding to the reconstituted vesicles, similar conditions were used, however, the UCP concentration was lower. Vesicles of 100 μL (of about 3.5 μg of protein) were suspended in buffer pH 6.8 containing 2% Triton X-100 at a final volume of 125 μL.

The binding rate of GTP was measured with an automated rapid mixing and separating sampling machine developed in our laboratory. UCP (0.16 mg/mL) suspended in 368 μL of Mes-Mops buffer (10 mM) and 0.5 mM PMSF were sucked into a 500 μL multipette syringe which was kept at 15 °C using a thermostated water jacket. After 1 min, the suspension was rapidly injected into a cup which contained 16 μL of 0.24 mM [^{14}C]GTP for a final concentration of 10 μM. The mixture was immediately sucked back into the multipette syringe causing rapid mixing. At predetermined time intervals, aliquots of 50 μL were injected stepwise onto small anion exchange columns (5 × 30 mm) containing 50 mg of Dowex. The injected aliquots were rapidly sucked through the columns by vacuum and the columns were washed with 100 μL of H_2O. The bound [^{14}C]GTP was determined by scintillation counting of the eluate. The time intervals were 2, 6, 20, 60, and 300s.

H^+ Influx Measurement. H^+ conductance was recorded using pyranine fluoresence at λ_{exc} = 467 nm and λ_{em} = 510 nm. Vesicles of 50 μL loaded with 100 mM potassium phosphate, pH 7.6, were diluted in a cuvette (5 × 5mm) with 280 μL of an isoosmolar buffer containing sucrose 0.28 M, Hepes 0.5 mM, EDTA 0.2 mM, and 1 μM pyranine, pH 7.5, giving 1.3 mg/mL of phospholipid and 2.5 μg/mL of protein at 10 °C. After the addition of lauric acid (125 μM) and the adjustment of the pH to 6.9 by the addition of H_2SO_4 in steps over 20 nmol H^+, transport was initiated by the addition of 2.5 μM valinomycin. The total H^+ uptake capacity was recorded after addition of 1 μM CCCP.

Cl^- Influx Measurement. Cl^- transport was measured by fluorescence of MQAE-loaded proteoliposomes at λ_{exc} = 355 nm and λ_{em} = 460 nm. A 50 μL aliquot of liposome suspenion (0.42 mg of phospholipid and 0.83 μg of protein) was added to 335 mL of sodium phosphate buffer (4 mM) containing 155 mM KCl. Cl^- influx was initiated by the addition of 2 μM valinomycin and the rate (J_{Cl}) in units of millimolar per minute was determined using a two-point calibration method as described by Verkmann et al. (*19*). The fluorescence corresponding to zero initial chloride concentration (F_i) was measured before the addition of valinomycin. The fluorescence (F_S) corresponding to the external Cl^- concentration (155 mM) was obtained at the end of every experiment by addition of 40 μM tributyltin acetate which caused complete equilibration with external Cl^-. J_{Cl} was calculated from the measured initial slope of the fluorescence decrease [dF(0)/dt], F_i, F_S, and the known final chloride concentration $[Cl]_S$ from the equation

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

K_{Cl} (0.15 mM^{-1}) was determined from the Stern−Volmer relation: $F_o/F = 1 + K_{Cl}[Cl]$, where F_o and F are fluorescence in the absence and presence of Cl^-. MQAE (50 μM) was quenched with increasing concentration of chloride in the same buffer with empty vesicles (1.09 mg of phospholipid/mL). To refer J_{Cl} to UCP protein, J_{Cl} was multiplied by intraliposomal volume per milligram of protein.

Miscellaneous. Protein concentration was determined by the method of Lowry et al. (*20*) in the presence of 1% sodium dodecyl sulfate (*21*) with BSA as Standard. SDS−PAGE was performed by the method of Laemmli (*22*) using 12.5% polyacrylamid. For immunoblotting, proteins on polyacrylamid gel were transfered to a nitrocellulose membrane. Detection was accomplished using rabbit polyclonal antiserum raised against UCP from hamster mitochondria and anti-rabbit IgG peroxidase conjugate. After 1 min incubation with chemiluminescence substrate (ECL Westernblotting, Amersham), the blot was exposed 30 s to film.

RESULTS

Isolation and Purification of UCP Expressed in Yeast. The level of heterologous expression of UCP in *S. cerevisiae* is qualitatively documented by the immunoblot in Figure 1. UCP was expressed 6 h after the induction by 0.4% galactose, as shown in the immunoblot of mitochondrial protein. Only a very small amount of UCP was present in mitochondrial protein before induction. The zero control is represented by cells which contained the expression vector without UCP-DNA.

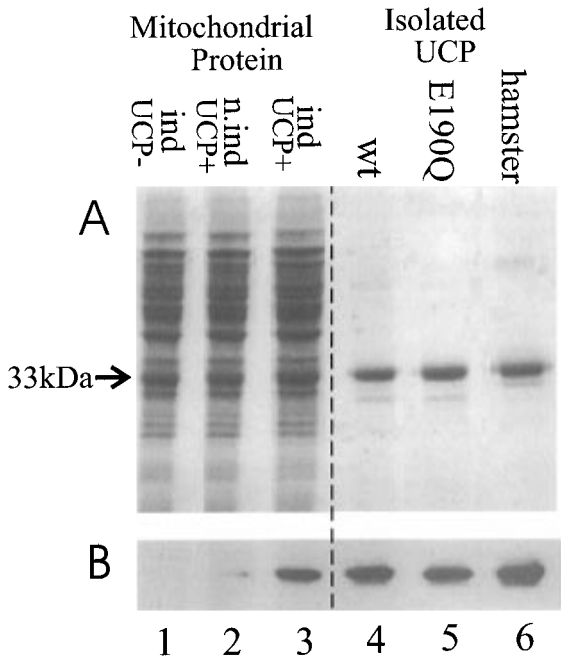

FIGURE 1: Analysis of expression and purification of wild-type and E190Q UCP. (A) SDS−PAGE, 12.5% polyacrylamide, and (B) immunoblot using antisera against UCP. Lanes 1−3, yeast mitochondria protein (30 μg) of cells grown (lane 1) with induced vector not containing UCP (ind UCP-), (lane 2) with not induced vector containing UCP-DNA (n.ind UCP+), (lane 3) with induced vector containing wild-type UCP-DNA (ind UCP+); lanes 4−6, isolated UCP (10 μg), (lane 4) wild-type UCP, (lane 5) E190Q UCP, (lane 6) UCP isolated from hamster mitochondria.

For quantitative determination of UCP in mitochondria, the titration with dansyl-GTP proved to be more reliable than [^{14}C]GTP binding, because a high background binding was measured in control mitochondria from the yeast cells without the plasmid carrying the UCP-DNA. On the basis of dansyl-GTP binding (see below), the level of expression was the same in wt and E190Q and was estimated to about 2.0−2.5% of the total protein content of the mitochondria. This value was obtained in a selective lactate medium containing yeast nitrogen base, whereas lower levels of UCP were measured in mitochondria from cells grown in complete (yeast extract, peptone) media, with either lactate or galactose as major carbon source.

From these mitochondria, UCP was isolated and purified for further studies. The solubilization and purification procedure were similar to those for hamster brown adipose tissue mitochondria and required solubilization with Triton X-100 in the presence of Na_2SO_4 followed by hydroxyapatite chromatography. In order to avoid copurification of yeast ADP/ATP carrier, room temperature and 4 times larger hydroxylapatite volumes were used than for extracts from hamster mitochondria. To further avoid contamination with the abundant mitochondrial porin in yeast, the ratio of detergent:mitochondrial protein was decreased to 1.2, since UCP is easier solubilized than porin (molecular mass = 29 kDa). As shown in the gels (Figure 1), the isolated proteins from wild-type and E190Q UCP do not contain any porin. The purity was estimated to 60−70%, based on the maximum binding of [^{14}C]GTP (see below), in comparison to the maximum binding of highly purified UCP from hamster (*4*). The yield of the isolated expressed UCP was about 1.2 to 1.5% of mitochondrial protein, both in wild-type and E190Q mutant.

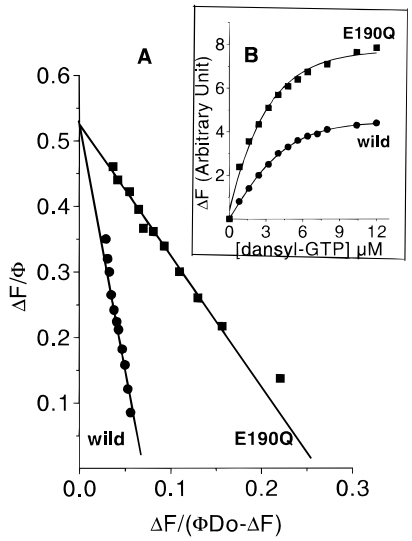

FIGURE 2: Fluorescence titration with dansyl-GTP of isolated wild-type and E190Q UCP at pH 7.5. UCP (35 μg/mL) in 20 mM Hepes buffer was titrated with dansyl-GTP at 10 °C, fluorescence 360−515 nm: (A) mass action plots [$\Delta F/\Phi = U_o - K_D\Delta F/(\Phi D_o - \Delta F)$ as described by Huang and Klingenberg (*6*)] where $\Delta F/\Phi$ = bound (micromolar) and $\Delta F/(\Phi D_o - \Delta F)$ = bound/free, and (B) titration curves. Fluoresence was recorded at λ_{exc} = 360 nm and λ_{em} = 515 nm.

Table 1: Dissociation constants (K_D) of wild-type and E190Q UCP expressed in S. cerevisiae at different pH

UCP	pH	[^{14}C]GTP[a] K_D (μM)	dansyl-GTP[b] K_D (μM)
wild-type	6.0	0.5	0.6
	6.8	1.6	2.1
	7.5	7.9	11.4
E190Q	6.0	1.0	1.2
	6.8	0.8	1.7
	7.5	2.5	3.4

[a] The K_D values were evaluated from [^{14}C]GTP titration of 200 μg/mL UCP on ice. [b] The K_D values were evaluated from fluorescence titration of 50 μg/mL at 10 °C (see Materials and Methods for titration measurements).

Binding of Nucleotides. The influence of the mutation on the binding site for nucleotides was assayed by using the fluorescent derivative dansyl-GTP and [^{14}C]GTP. The maximum binding with [^{14}C]GTP also served to determine the content of UCP. An example of the concentration dependence of dansyl-GTP binding to isolated wt and E190Q UCP is shown in Figure 2 by using the fluorescence increase in dependence of sequential additions of dansyl-GTP. A high pH has been chosen in order to demonstrate the difference between wt and mutant UCP. The mass action plot shows that E190Q has a higher affinity for GTP than wt. A similar result is obtained with [^{14}C]GTP (data not shown).

In view of the known pH dependence of the affinity for nucleotides, the K_D was determined at pH 6.0, 6.8, and 7.5 for both [^{14}C]GTP and dansyl-GTP. Table 1 lists the results of several binding experiments and titrations. For wt UCP expressed in yeast, the affinity for GTP is about the same as previously determined for UCP from hamster (*5*). Also, the K_D increases drastically from pH 6.0 to 7.5 with about 16-fold decrease in the affinity for [^{14}C]GTP as in UCP from hamster. With dansyl-GTP, a 19-fold raise of the K_D with the pH is determined. However, in E190Q, the K_D for [^{14}C]-GTP binding increased from pH 6.0 to 7.5 only 2.5-fold and 2.8-fold for dansyl-GTP binding. In conclusion, the neu-

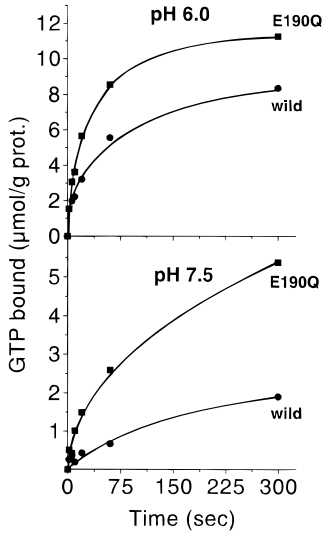

FIGURE 3: Time study of [^{14}C]GTP binding to isolated wild-type and E190Q UCP at pH 6.0 and 7.5 using kinetic measurements by the rapid removal procedure as described under Materials and Methods. At 15 °C, 0.16 mg of protein/ml incubated in 368 μl Mes-Mops buffer (10 mM) and 0.5 mM PMSF with 10 μM [^{14}C] GTP for the time interval indicated at pH 6.0 and 7.5. The bound [^{14}C]-GTP was determined by scintillation counting of the eluate from the anion exchange column. The time intervals were 2, 6, 10, 20, 60, and 300 s.

Table 2: Rates of Binding of [^{14}C]GTP to Wild-Type and E190Q UCP at Two pH[a]

pH	wild-type binding rate $k_{on} \times 10^{-3}$ (M^{-1} s^{-1})	E190Q binding rate $k_{on} \times 10^{-3}$ (M^{-1} s^{-1})
6.0	0.45	1.39
7.5	0.05	0.33

[a] The rate constants were evaluated from time study of [^{14}C]GTP binding to UCP at 15 °C.

tralization of E190 strongly decreases the pH-controlled changes of the affinity of nucleotide binding. As a result, in E190Q at pH 7.5 the affinity for nucleotides is much higher than in wt UCP, although at pH 6.0 it is even slightly lower.

Binding Rates. We have previously shown that the changes in the K_D are largely due to changes in the binding rate rather than dissociation rates (*6*). Further, it was shown that these binding rates are unusually slow and can be resolved by sampling, and separation techniques. Accordingly, the binding rate of [^{14}C]GTP to isolated UCP from yeast was measured by an automated mixing, sampling and separation apparatus (Figure 3). The time progress curves of the binding clearly illustrate the faster binding rates to E190Q than to wt UCP. The evaluation according to a second-order reaction gives rate constants listed in Table 2. Whereas from pH 6.0 to 7.5 the binding rates decreased 10-fold for the wild type, they decreased only about 4 times with the E190Q mutant. As a result, at pH 7.5 the binding rate for E190Q is found to be 7 times faster than that to wt UCP.

The fluorescent GTP derivative (dansyl-GTP) enables direct recordings of the binding to UCP. Thus, the changes in the binding characteristics caused by the E190Q mutation are visualized. In these recordings (Figure 4), not the total fluorescence but the decrease caused by addition of excess ATP, which displaces the dansyl-GTP, is taken as the specific

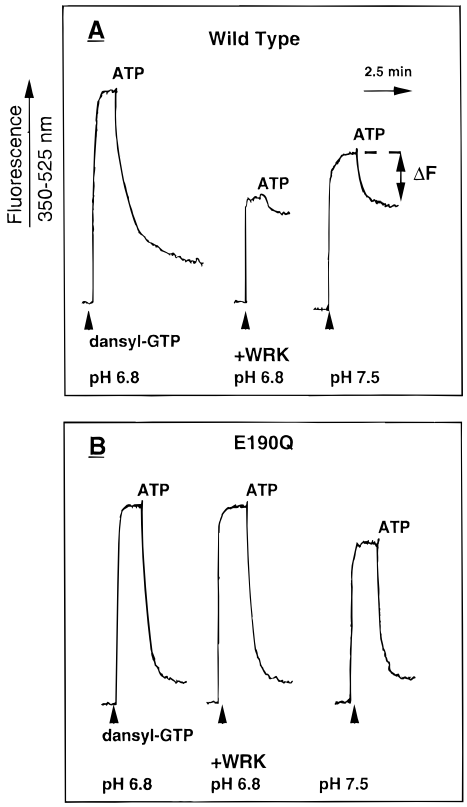

FIGURE 4: Effect of WRK reagent (25 μM) and pH on the fluorescence response of dansyl-GTP binding to wild-type (A) and E190Q (B) UCP. Dansyl-GTP (10 μM) was added to a solution of 33 μg/mL UCP in 20 mM Hepes buffer at 10 °C. After fluorescence equilibrium, 1.5 mM ATP was added to displace the fluorescent ligand from the binding sites. Fluoresence was observed at λ_{exc} = 350 nm and λ_{em} = 525 nm.

fluorescence associated with the binding to UCP. Both with wt and E190Q UCP, about the same decrease of fluorescence ΔF is measured by addition of ATP at low pH. While at pH 6.8 the recordings with the wt and E190Q UCP are similar, striking differences are observed by shifting the pH to 7.5, where in the wild-type, ΔF is strongly diminished, but there is no change of ΔF in the E190Q mutant in relation to the total fluorescence. In other words, the dansyl-GTP binding in the E190Q mutants has lost largely its pH sensitivity.

Another drastic difference is the response to the carboxyl reagent WRK. Pretreatment of UCP with WRK has been shown to abolish nucleotide binding in UCP from hamster mitochondria. Further, WRK was shown to incorporate into E190. Here, the fluorescence of dansyl-GTP is used to monitor the effects of WRK on nucleotide binding. As shown in Figure 4, with UCP expressed in yeast, a nearly complete suppression of dansyl-GTP binding by WRK is observed. After ATP addition, the fluorescence decrease amounts to only 10% of the untreated UCP. In contrast, with E190Q UCP, pretreatment of WRK is virtually without any effect on the dansyl-GTP binding. We also assayed the effect of WRK on [^{14}C]GTP binding to wt and mutant UCP (Table 3). Again, low amounts (25 μM) of WRK suppress GTP binding to 70% on wt but have no effect on E190Q UCP. In summary, the E190Q mutation has eliminated completely the interaction of WRK with the nucleotide binding site in UCP.

H^+ and Cl^- Transport. In order to test the influence of the mutation on the basic transport properties of UCP, i.e.,

Table 3: Effect of WRK Reagent on Dansyl-GTP and [^{14}C]GTP Binding to Wild-Type and E190Q UCP

UCP	WRK	dansyl-GTP bound (Δfluorescence)[a]	[^{14}C]GTP bound (μmol/g of protein)[b]
wild-type	−	12.4	9.5
	+	1.5	3.3
E190Q	−	11.6	9.6
	+	11.5	9.3

[a] Dansyl-GTP was added to a solution of UCP (33 μg/mL) in 20 mM Hepes buffer, pH 6.8, containing WRK reagent (25 μM) at 10 °C. ΔF is the specific fluorescence (arbitrary unit) obtained by subtracting from the total fluorescence the unspecific fluorescence measured in the presence of 1.5 mM ATP. [b] WRK reagent (25 μM) was added to a solution of UCP (200 μg/mL) in 15 mM Mops buffer, pH 6.5, on ice for 20 min, before incubation with [^{14}C]GTP (15 μM) for another 10 min.

transport of H^+/OH^- and Cl^-, isolated UCP was reconstituted into phospholipid vesicles. H^+ transport was measured using pyranine as a fluorescent pH sensor, and Cl^- transport was followed using MQAE as a Cl^- sensitive fluorescence probe. To check whether the mutation affected the incorporation of UCP into vesicles, [^{14}C]GTP binding was measured at the reconstituted proteoliposomes. Both for E190Q as well as wt UCP, 70−80% of UCP offered was incorporated into the vesicles.

H^+ transport was activated by addition of 125 μM lauric acid and was measured at pH 6.9 and 7.5. Our main interest was to look for the changes of inhibition by mutation. Because the pH sensitivity of GTP and GDP binding to UCP was shown to be quite different, both inhibitors were applied here for assaying the pH dependence of inhibition in E190Q versus wt UCP. The results of the H^+-uptake rate measurements are summarized in Figure 5. There is no major difference of the uninhibited H^+ transport activity between wt and E190Q UCP. In agreement with earlier data, at pH 6.9, H^+ transport is already inhibited to more than 50% at 5 μM GTP and to about 40% at 5 μM GDP and 80% at 100 μM GTP and GDP. The residual activity is probably due to UCP inserted into the vesicles inside-out where the binding site is inaccessible for the nucleotides. Since the degree of inhibition is equal for wt and E190Q, we can conclude that both are incorporated with a similar sidedness. The data also show that at nonsaturating 5 μM GTP and GDP, H^+ transport is more inhibited in E190Q than in wt UCP. At pH 7.5, the sensitivity toward nucleotides in wt UCP is drastically decreased; GTP (5 μM) does not inhibit at all and GDP at the same concentration only to 20%. The inversion of the sensitivity toward GTP versus GDP on increasing the pH is in line with a much stronger pH dependence of GTP binding reported previously (5). Most significantly at this high pH, H^+ transport by E190Q is inhibited to 60−70% with 5 μM GTP and GDP and thus to about the same extent as at pH 6.9.

For measuring Cl^- influx, the vesicles were loaded with MQAE as described in Materials and Methods. The uptake rates of Cl^- are the same for wt and E190Q UCP (Figure 6). At pH 6.8, Cl^- transport is inhibited by 5 μM GDP and GTP to 80% in wt and to 85% in E190Q. There is no difference at saturation with 100 μM GTP or GDP. At pH 7.5, in wt UCP, 5 μM GTP is unable to inhibit and 75 μM GDP only at 15%, whereas, in E190Q, the inhibition by both 5 μM GTP and GDP reaches 70%. At this pH, Cl^- transport in wt UCP is not even fully inhibited by 100 μM GTP because 155 mM Cl^- competes with GTP binding, whereas in E190Q transport is inhibited by 100 μM GTP and GDP to the maximum of 80%. In conclusion, Cl^- transport

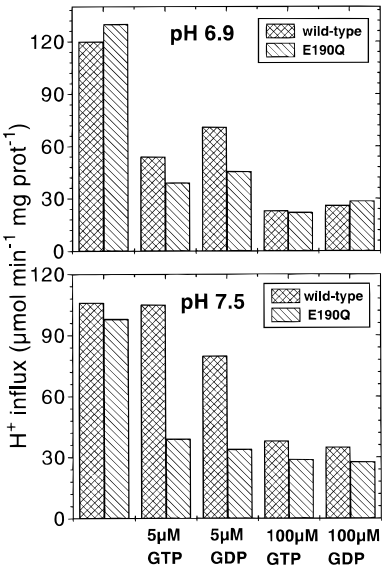

FIGURE 5: Proton influx into phospholipid vesicles reconstituted with purified wild-type or E190Q UCP at pH 6.9 and 7.5. H^+ influx was measured as the change in external pH monitored by pyranine fluoresence at $\lambda_{exc} = 467$ nm and $\lambda_{em} = 510$ nm. A 50 μL portion of vesicles was added to 0.5 mM Hepes buffer pH 8.0 containing 1 μM pyranine, 0.5 mM EDTA, and 280 mM sucrose to a final volume of 330 μL at 10 °C. Valinomycin (Val) 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 or 7.5. The uncoupler CCCP (1 μM) was used to determined the capacity of H^+ conductance across the vesicles.

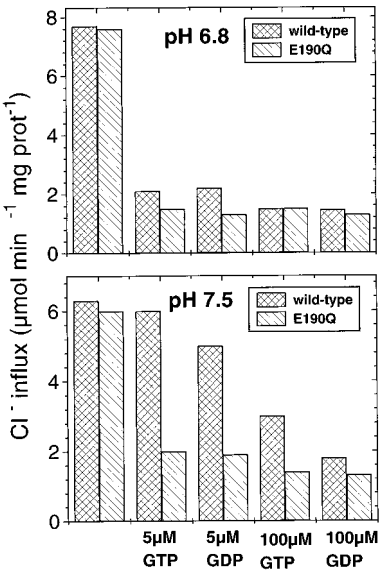

FIGURE 6: Chloride transport into phospholipid vesicles reconstituted with purified wild-type or E190Q UCP at pH 6.8 and 7.5. Cl^- influx was monitored by fluoresence of MQAE loaded into the vesicles at $\lambda_{exc} = 355$ nm and $\lambda_{em} = 460$ nm. A 50 μL portion of vesicles was suspended in 4 mM sodium phosphate buffer containing 155 mM KCl to a final volume of 385 μL at 10 °C. The chloride influx rate was monitored after addition of 2 μM valinomycin as described under Materials and Methods. Near the completion of the fluorescence experiment, tributyltin acetate (40 μM) was added to equilibrate internal and external chloride. The influx rates in units of micromoles per minute milligram of protein were calculated from the fluorescence data by using eq 1.

dramatically demonstrates the increased sensitivity of E190Q toward nucleotides as compared to wt UCP, particularly at pH >7.0.

DISCUSSION

The expression of UCP in *S. cerevisiae* from rat for mutagenesis has been successfully performed in other laboratories (*23*, *24*). We chose to clone the UCP from hamster since our long-term research starting with the first isolation of UCP had concentrated on this source. For the expression of UCP from hamster in yeast, we have used a similar system as previously reported for UCP from rat. Our expression level of 2.0−2.5% UCP of the total protein content of the mitochondria approximately agrees with that reported by Rial et al. (*25*), but is substantially lower than that of 10% UCP/protein claimed by Freeman and Garlid (*26*). However, their claim is not substantiated by their data from which one calculates at most 2.5% based on the reported binding of GDP to the mitochondria. In all these cases, expression of UCP is induced by galactose. Various media were used for the growth of the yeast, where an artificial nitrogen base medium was found to give maximum UCP expression.

The first use of mutagenesis of UCP in our hands was directed toward a residue which we had previously suggested to be a pH sensor for the regulation of nucleotide binding to UCP (*8*). According to our model, the ionization of a critical carboxyl group modulates nucleotide binding (*5*, *6*). In the preceding work, the existence of a carboxyl was verified and identified as E190 using Woodward reagent K (*8*). Taking into account this result we should predict according to our model that the elimination of the carboxyl group E190 should change the pH dependence of nucleotide binding and of the associated inhibition of H^+/OH^- transport. More specifically, the removal of the carboxyl group by conversion of glutamic acid 190 into glutamine should increase the binding affinity, particularly at pH >6.9.

The results reported in this paper confirm exactly this prediction and thus vindicate the role of E190 as a pH sensor in nucleotide binding. Although at pH 6.0 the binding affinity of GTP to E190Q is only half as large as to wt, at pH 7.5 the affinities are reversed, i.e., to E190Q about 3 times higher than to wt. These differences also appear when titration with the fluorescent derivative dansyl-GTP was performed at pH 7.5. These mutational alterations are further reflected in the kinetics of binding of [^{14}C]GTP. Whereas in the wild-type the binding rate is decreased 10-fold from pH 6 to 7.5, it decreases only about 4-fold in the E190Q mutant. The fluorescence of dansyl-GTP also shows that the dramatic decrease of dansyl-GTP binding to wt UCP is largely abolished in the E190Q by raising the pH.

The carboxyl reagent WRK has been instrumental in suggesting E190Q as a pH-controlling carboxyl group (*8*). WRK abolishes nucleotide binding by attacking specifically E190. Therefore, the prediction would be that the E190Q mutation prevents the suppression of the nucleotide binding by WRK. This is exactly what we observed here. The binding of either [^{14}C]GTP or dansyl-GTP was fully resistent to WRK in E190Q.

The dramatic changes in the sensitivity toward nucleotide binding by the E190Q mutation were also demonstrated on the level of the transport activity for H^+ and Cl^-. At pH 7.5, H^+ transport was much more sensitive to both GTP or GDP in E190Q than in wt. This was still more evident for Cl^- transport. It shows that E190 is involved in the pH regulation of inhibition both of H^+ and Cl^- transport. E190Q largely restitutes the inhibition by GTP and GDP at pH >7. There is a significant difference in the response to pH upshift of the inhibition both of H^+ and Cl^- transport by GTP versus GDP. In wt at pH 6.8, GTP inhibits stronger than GDP, and at pH 7.5, this relation is reversed. This difference of inhibition is completely eliminated in E190Q.

These results confirm the concept that nucleotide binding is controlled by a carboxyl group both for the nucleoside di- and triphosphates. As one of the two intrahelical carboxyl groups in UCP, E190 might have been a candidate for participating in H^+/OH^- translocation. The H^+ and Cl^- transport activities are fully retained, showing that E190 is not involved in transport. Also, the activation by fatty acids of H^+ transport is not changed by the removal of this carboxyl.

The persistence of high binding affinity at pH >7.0 in the E190Q mutant vindicates what has been a fairly bold extrapolation from the pK_D-pH analysis of nucleotide binding, i.e., the critical role of a carboxyl group in UCP and its interplay with the given H^+ dissociation of nucleotides. In those relatively rare cases where a nucleotide binding site is selective for the free non-Mg^{2+} complexed nucleotides, the pH should influence binding around the p$K \approx 6.5$ of the γ-phosphate. It is reasonable to assume that the fully ionized forms NDP^{3-} or NTP^{4-} exhibit stronger binding than $NDPH^{2-}$ or $NTPH^{3-}$. Thus, what we observed with E190Q is a pH dependence of binding, which is not superimposed by this carboxyl group but largely dominated by the pK of the nucleotide γ-phosphate. With this reasoning we should actually expect a decrease of affinity at lower pH <6.5 in E190Q, which was not confirmed by our measurements (not shown). So, further H^+ dissociating groups could be involved. In the closely related ADP/ATP carrier, ATP and ADP also bind as the free nucleotides (*27*). Binding to the isolated ADP/ATP carrier is very difficult to measure because of the low affinity. The pH dependence of the transport rate with the reconstituted ADP/ATP carrier has a clear maximum between 6.8 and 7.2 and decreases strongly with pH <6.8 (*28*, *29*). Also here, obviously the fully ionized forms ATP^{4-} and ADP^{3-} are those which actively interact with the carrier.

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REFERENCES

1. Nicholls, D. G. (1979) *Biochim. Biophys. Acta 549*, 1−29.
2. Klingenberg, M. (1990) *Trends Biochem. Sci. 15*, 108−112.
3. Locke, R. M., Rial, E., Scott, I. D., and Nicholls, D. G. (1982) *Eur. J. Biochem. 129*, 373−380.
4. Lin, C. S., and Klingenberg, M (1982) *Biochemistry 21*, 2950−2956.
5. Klingenberg, M. (1988) *Biochemistry 27*, 781−791.
6. Huang, S.-G., and Klingenberg, M. (1995) *Biochemistry 34*, 349−360.

7. Huang, S.-G., and Klingenberg, M. (1996) *Biochemistry 35*, 7846−7854.
8. Winkler, E., Wachter, E., and Klingenberg, M. (1997) *Biochemistry 36*, 148−155.
9. Bernardi, G. (1971) *Methods Enzymol. 22*, 325−339.
10. Winkler, E., and Klingenberg, M. (1994) *J. Biol. Chem. 269*, 2508−2515.
11. Sambrock, J., Fritsch, E. F., and Maniatis, T. (1989) in *Molecular Cloning: A Laboratory Manual* (Nolan, C., Ed.) Cold Spring Harbor Laboratory, Plainview, NY.
12. Cesareni, G., and Murray, J. A. H. (1987) in *Genetic Engineering: Principles and Methods* (Seltow, J. K., Ed.) Vol. 9, pp 135−154, Plenum Press, New York.
13. Stearns, T., Ma, H., and Botstein D. (1990) *Methods Enzymol. 185*, 280−296.
14. Gawaz, M., Douglas M. G., and Klingenberg M. (1990) *J. Biol. Chem. 265*, 14202−14208.
15. Lin, C. S., and Klingenberg M. (1980) *FEBS Lett. 113*, 299−303.
16. Klingenberg, M., Winkler, E., and Huang, S.-G. (1995) *Methods Enzymol. 260*, 369−389.
17. Fiske, C. H., and Subbarow, Y. (1925) *J. Biol. Chem. 66*, 375−400.
18. Huang, S.-G., and Klingenberg M. (1995) *Eur. J. Biochem. 229*, 718−725.
19. Verkman, A. S., Takla, R., Sefton, B., Basbaum, C., and Widdicombe, J. H. (1989) *Biochemistry 28*, 4240−4244.
20. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem. 193*, 265−275.
21. Schagger, H., and Von Jagow, G. (1987) *Anal. Biochem. 166*, 368−379.
22. Laemmli, U. K. (1970) *Nature 277*, 680−684.
23. Murdza-Inglis, D. L., Patel H. V., Freeman K. B., Jezek P., Orosz, D. E., and Garlid, K. D. (1991) *J. Biol. Chem. 266*, 11871−11875.
24. Arechaga, I., Raimbault, S., Prieto, S., Levi-Meyrueis, C., Zaragoza, P., Miroux, B., Riquier, D., Bouillaud, F., and Rial, E. (1993) *Biochem. J.* 296, 693−700.
25. González-Barroso, M., Fleury, C., Arechaga, I., Zaragoza, P., Levi-Meyrueis, C., Raimbault, S., Riquier, D., Bouillaud, F., and Rial E. (1996) *Eur. J. Biochem. 239*, 445−450.
26. Murdza-Inglis, D. L., Modriansky, M., Patel, H., Woldegiorgis, G., Freeman, K. B., and Garlid, K. D. (1994) *J. Biol. Chem. 269*, 7435−7438.
27. Pfaff, E., and Klingenberg, M. (1968) *Eur. J. Biochem. 6*, 66−79.
28. Krämer, R., and Klingenberg, M. (1980) *Biochemistry 19*, 556−560.
29. Klingenberg, M., Grebe, K., and Appel, M. (1982) *Eur. J. Biochem. 126*, 263−269.

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