In the Uncoupling Protein (UCP-1) His-214 Is Involved in the Regulation of Purine Nucleoside Triphosphate but Not Diphosphate Binding*

(Received for publication, May 11, 1998, and in revised form, July 7, 1998)

Karim S. Echtay, Martin Bienengraeber, Edith Winkler, and Martin Klingenberg‡

From the Institute of Physical Biochemistry, University of Munich, Schillerstrasse 44, 80336 Munich, Germany

The nucleotide binding to uncoupling protein (UCP-1) of brown adipose tissue is regulated by pH. The binding pocket of the nucleoside phosphate moiety has been proposed to be controlled by the protonization of a carboxyl group (pK ~4.5) for both nucleoside diphosphates (NDP) and nucleoside triphosphates (NTP) (identified as Glu-190) and of a histidine (pK ~7.2) for NTP only. Here we identify His-214 as a pH sensor specific for NTP binding only. In reconstituted UCP-1 from hamster, DEPC diminishes binding of NTP but not of NDP. It also prevents inhibition of H⁺ transport by NTP but not by NDP. Hamster UCP-1 expressed in Saccharomyces cerevisiae was mutated to H214N resulting in only moderate change of the binding affinity for NTP (GTP) but a 10-fold affinity decrease with the bulkier substituent in H214W, whereas the affinity for NDP (ADP) was largely unchanged. The steep decrease with pH of the binding affinity for NTP in wild type (from pH 6.0 to 7.5) was much flatter in the mutants. Also, the pH dependence of binding and dissociation rates was diminished in these mutants. The transport of H⁺ and Cl⁻ was not affected. Thus, His-214 is only involved in nucleotide binding, whereas, as previously shown, His-145 and His-147 are involved only in H⁺ transport. The results validate the earlier proposal of a histidine regulating the NTP binding in addition to a carboxyl group controlling both NTP and NDP binding. It is proposed that His-214 protrudes into the binding pocket for the γ-phosphate thus inhibiting NTP binding and that His214H⁺ is retracted by a background –CO₂⁻ group to give way for the γ-phosphate.

The uncoupling protein (UCP-1) from brown adipose tissue short circuits H⁺/OH⁻ generated by the respiratory chain, thus releasing heat from the oxidation of substrates (1, 2). The H⁺/OH⁻ transport activity of UCP is regulated by fatty acids as activators and by purine ribose nucleotides as inhibitors (1, 3). The inhibition by nucleotides (ATP, ADP, GTP, and GDP) is further regulated by the H⁺ concentration. With higher pH, the Kᵣ values for H⁺ transport inhibition and the Kᵣ values for nucleotide binding increase (4, 5). This affinity decrease with pH is stronger with nucleoside triphosphates (GTP and ATP) than with the diphosphates. From the detailed analysis of the pKᵣ/pH relation, a model was derived in which two H⁺ dissociating groups in the binding pocket of UCP are controlling the nucleotide binding (4, 5). It was proposed that the protonation of an acidic residue (Glu or Asp) is prerequisite for binding both NDP and NTP and in addition the protonation of a His residue for NTP only. The dual pH control of NTP binding was regarded to be critical for relieving nucleotide binding within a narrow pH increase and thus to activate H⁺ transport.

Recently, we have been able to verify the existence of a –CO₂⁻ group as a pH sensor for nucleotide binding by using Woodward reagent K. This group was identified as Glu-190, which is located in the fourth transmembrane helix of UCP (6). Furthermore, by using an expression system for UCP in Saccharomyces cerevisiae, we demonstrated that by mutagenic substitution of Glu-190 the affinity of nucleotide binding increased and became largely pH-independent (7). Thus the role of Glu-190 as a pH sensor for nucleotide binding in UCP was established. This encouraged the search for a His residue as a second pH sensor. Here we report the identification by mutagenesis of His-214 as a critical group participating in the pH control of NTP binding. The results provide evidence that sterical factors play a major role in the pH-dependent differentiation between nucleoside triphosphate versus diphosphate binding. In the neutral form His-214, suggested to be located in the binding pocket of the γ-phosphate, is concluded to protrude into the binding pocket, whereas in the charged state it is retracted by a background negative charge thus making space for the γ-phosphate. This pH-controlled movement of the His-214 is superimposed by the pH control of Glu-190 to produce the strong pH dependence of the nucleoside triphosphate binding, which should be of great importance for the regulation of the uncoupling activity of UCP.

EXPERIMENTAL PROCEDURES

Materials—n-Decylpentaoxyethylene (C₁₀E₅), diethylpyrocarbonate, and Dowex 1-X8 (200–400 mesh) were obtained from Fluka. [¹⁴C]GTP, [¹³C]ADP, and [³H]GDP were from Amersham Corp. 2'-'O-2'-O-Dansyl-GTP was synthesized as described by Huang and Klingenberg (5). The fluorescence dyes MQAE and pyranine (8-hydroxyquinine-1,3,6-trisulfonic acid, trisodium salt) were purchased from Molecular Probes.

Mutagenesis—The gene coding for UCP-1 from hamster was cloned in pEEMBLY4 vector (8) under the control of the gal10/cyc1 promoter as described previously (7, 9). The histidine mutant was generated by using an oligonucleotide-directed system (USE Mutagenesis Kit, Pharmacia). The CAT codon for His-214 was changed to the AAC and TGG cordon for asparagine and tryptophan to construct H214N and H214W, respectively. The sequence of the mutant was verified by DNA sequencing, also checking for the absence of any other mutation in the UCP-coding frame. The S. cerevisiae strain W303 was transformed with a
plasmid containing the mutation. Yeast transformants were grown in selective lactate medium, and expression was induced by adding 0.4% galactose.

**Isolation of Mitochondria and Determination of UCP Content—**Mitochondria were isolated from yeast by differential centrifugation following a procedure previously described (7). 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 dansyl-GTP fluorescence titration. To remove endogenous residual bound nucleotide, mitochondria were treated with Dowex as mentioned previously (7).

Isolation, Purification, and Reconstitution of UCP—UCP was isolated from yeast mitochondria using Triton X-100 as detergent according to the protocol described for hamster brown adipose tissue mitochondria (10) with modification as reported previously (7). Purified UCP was reconstituted into phospholipid vesicles following a protocol previously described (7). Briefly, UCP was suspended with egg yolk phosphatidylcholine (phospholipid:protein = 500:1 by mass), a medium of 100 mM potassium phosphate, pH 7.6, for H+ transport or 100 mM sodium phosphate, pH 6.8, for Cl− transport measurement in addition to 0.2 mM EDTA and 1 mM PMSF, and C10E5 (detergent:phospholipid = 1.4 by mass). Vesicle formation was accomplished by slow removal of the detergent with Bio-Beads SM-2 at 4 °C. The external solute was removed passing the vesicle over a G-75 column. Vesicles for Cl− transport measurement were loaded with MQAE through diffusion for 17 h in the dark at 4 °C.

**Reconstitution and H+ Transport Measurement Used for the Chemical Modification by DEPC—**UCP was isolated from mitochondria of brown fat adipose tissue and reconstituted into phospholipid vesicles as described previously (11). For H+ uptake measurements, vesicles were loaded with 100 mM potassium phosphate and 0.4 mM EDTA, pH 7.5. Vesicles with a phospholipid concentration of 19 and 0.124 mg/ml protein were treated with increasing concentrations of DEPC (0 to 2.2 mM) resulting in DEPC:His ratio of 0–150 mol/residue. After modification by shaking for 1 h at 8 °C, the external solutes were removed from the proteoliposomes by Sephadex G-75 column, and the H+ transport measurements were performed as described (11) using a low impedance combination pH electrode.

**Fluorescent Measurements Used for H+ and Cl− Transport—**For UCP expressed in yeast, H+ uptake activity was measured on an MFP-44A fluorescence spectrophotometer using pyranine fluorescence at λem = 467 nm and λ exc = 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 lauryl arachidic 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 λem = 355 nm and λ exc = 460 nm in a medium containing 4 mM sodium phosphate, pH 6.8, and 155 mM KCl. Cl− influx was initiated by the addition of valinomycin (2 μM), and the rate (dCl−/dt) was determined using a two-point calibration method as described by Verkman et al. (12) as shown in Equation 1.

\[ J_{Cl} = \frac{(F_t - F_i)R_C + 1/RC_i}{L} \times [dF(t)/dt] \]  

(Eq. 1)

The applied methods for transport measurements resembled those described previously (7).

**Nucleotide Binding Measurements—**Binding titration with [14C]GTP and [14C]ADP followed in principle the published procedure using Dowex for removal of free radiolabeled nucleotide (4, 7). The binding rate of [14C]GTP was measured with an automated rapid mixing and separating sampling machine developed in our laboratory as described previously (7). Nucleotide fluorescence derivative (dansyl-GTP) was applied for GTP dissociation rate determination. The dissociation was followed by the fluorescence decrease on addition of large excess ATP after fluorescence equilibrium of dansyl-GTP binding to UCP at λex = 350 nm and λem = 525 nm. The buffer used was 20 mM HEPES or MES at 15 °C.

**RESULTS**

**Influence of the His Reagent Diethyl Pyrocarbonate—**For elucidating the proposed role of histidine in nucleotide binding, we applied the amino acid reagent diethyl pyrocarbonate (DEPC), which is reported to react primarily with histidine (13). In experiments with hamster BAT mitochondria, the reagent inhibited nucleotide binding only at very high concentrations where also reactions with primarily amino groups and —SH groups occur. With isolated UCP, DEPC at a critical concentra-

---

**Fig. 1. Effect of the histidine reagent, DEPC, on H+ transport and its inhibition by nucleotides as well as on the nucleotide binding.** The three curves represent H+ transport rates without (●) or with 30 μM (●) and GTP (●). UCP containing proteoliposomes loaded with 100 μM potassium phosphate, pH 7.5, was incubated with increasing concentrations of DEPC resulting in DEPC:His ratio of 0–150 mol/residue for 1 h at 8 °C as described under "Experimental Procedures." The vesicles were passed over Sephadex G-75, and H+ uptake rate was measured without or with 30 μM GTP or GDP. For nucleotide binding measurements, aliquots of 100 μl proteoliposomes buffered with 10 mM ammonium acetate, pH 6.0, were incubated with 10 μM [14C]GTP or [3H]GDP for 30 min at 0 °C. After shaking with 10 mg Dowex 1-X8 (200–400 mesh, Cl− form) for 10 s, the anion exchanger was removed rapidly by filtration. The pass-through represents the radiolabeled nucleotide bound to UCP.

---

**Characterization of His-214 Mutant UCP Expressed in S. cerevisiae—**The use of dansyl-GTP titration allows quantification of H214N and H214W UCP incorporation into mitochondria which is estimated to about 2.5% of the total protein content similar to that of wt. Fig. 2 shows the SDS gel and immunoblot of isolated UCP and mitochondria, respectively. The isolated proteins from wt and His-214 mutant UCP are largely freed from two main contaminants, namely porin and ADP/ATP carrier. The yield of isolated wt and H214W UCP is 1.2–1.5% of total mitochondrial protein, but only 0.5–0.7% for H214N UCP which is half the amount obtained as compared with wt. The purity of the isolated wt and H214W protein is estimated to about 70% based on maximum binding of [14C]GTP to compare to maximum binding of highly purified UCP from hamster. Since the binding capacity of the isolated H214N UCP is 40% less (as shown below), the purity of the isolated protein is estimated the same as the wt based on SDS gel comparison.

H+ and Cl− Transport—H+ uptake in proteoliposomes reconstituted with purified UCP requires a K+ diffusion potential negative inside, generated by the valinomycin-catalyzed efflux of K+ and high buffering capacity inside. Cl− uptake is induced
by a K+ diffusion potential positive inside, generated by the valinomycin-induced influx of K+. The rates of H+ and Cl− uptake are summarized in Fig. 3. Proteoliposomes reconstituted with H214W transport H+ and Cl− at the same rate as wt, but with H214N only to about 60% of wt. Treatment of the proteoliposomes with 100 μM GTP or GDP leads to 85% inhibition of transport activities for both wt and His-214 mutant UCP. The residual activity may be ascribed to inversely inserted UCP molecules, whose binding sites cannot be accessed by the nucleotides. The 40% lower transport activity of H214N mutant UCP in is line with the same percentage decrease in nucleotide binding capacity. Thus, the turnover of H214N in H+ and Cl− transport is the same as wt UCP. We can conclude that His-214 is not involved in the transport activities of UCP.

Nucleotide Binding—To study the binding to isolated UCP of nucleoside tri- and diphosphate and its pH dependence, titration with [14C]GTP and [14C]ADP using the Dowex method (see “Experimental Procedures”) was performed at different pH values. GDP had to be replaced by ADP, as a purine nucleoside diphosphate, since no [14C]GDP available. The mass action plot evaluated from the concentration dependence with [14C]GTP (Fig. 4) gives a $B_{max} = 12 \, \mu$mol/g of protein of GTP bound for wt, 15 μmol/g of protein for H214W, and 8 μmol/g of protein for H214N UCP. Thus the binding capacity is increased by 25% in H214W and reduced by 40% in H214N as compared with wt on isolation of UCP, whereas the binding determined for mitochondria is the same in the mutants and wt. A similar percentage variation is obtained with [14C]ADP. Table I lists the $K_D$ values of several binding experiments and titrations at pH 6.0, 6.8, and 7.5 for both [14C]GTP and [14C]ADP. The $K_D$ for [14C]GTP of wt increases 8-fold from 1.05 μM at pH 6.8 to 7.9 μM at pH 7.5, whereas in His-214 mutants a slight change in the affinity is observed with only 1.5-fold increase of $K_D$ in H214W and almost no variation in H214W from pH 6.8 to 7.5. In contrast to the low pH sensitivity shown with nucleoside triphosphate (GTP), the affinity of His-214 mutant to nucleoside diphosphate (ADP) is highly pH-sensitive. As shown in Table I and Fig. 4, the $K_D$ value for [14C]ADP increases by shifting the pH from 6.0 to 7.5 in H214W by the same factor, i.e., ~12 times as in wt and ~18 times in H214N.

Binding and Dissociation Rates—In order to elucidate further the involvement of His-214 on the pH regulation of nucleoside triphosphate binding, a kinetic study of [14C]GTP binding was performed. Binding and dissociation rate determinations supplement the equilibrium measurements and can reflect changes in affinity under conditions, e.g., low or very high affinity, where direct $K_D$ determinations are difficult. Table II summarizes the rate constants ($k_{on}$) evaluated from the initial slope according to the second-order reaction at different pH values. The $k_{on}$ for the wt UCP decreases about 7-fold, from pH 6.5 to 7.5. For the two His-214 mutants, on the other hand, the time progress curves (Fig. 5) of binding clearly illustrate the slower binding at low pH and the faster binding rate at high pH as compared with wt. The $k_{on}$ of H214N and H214W changes slightly (1.4-fold) from pH 6.5 to 7.5 as compared with a 7-fold decrease in the case of wt.

For measuring the dissociation rates, the fluorescent nucleotide derivative (dansyl-GTP) was used and evaluated from the fluorescence decrease upon removal of dansyl-GTP on addition of large excess ATP. Table II lists the dissociation rates ($k_{off}$) for wt, H214N, and H214W UCP at different pH values. The $k_{off}$ with wt UCP increases about 5-fold from pH 6.5 to 7.5, whereas with H214N and H214W the $k_{off}$ changes just about 1.4-fold. Remarkably, the dissociation rate with H214W is lower than with wt and H214N at low pH. The pH dependence of the binding and dissociation rate constants are plotted in Fig. 6. As measured with [14C]GTP, the binding rate for wt UCP decreases with pH according to $\Delta pK_{on}/\Delta pH \approx 1$, and the dissociation rate of dansyl-GTP increases with pH according to $\Delta pK_{off}/\Delta pH \approx -1$. For H214N and H214W UCP, the $k_{on}$ and $k_{off}$ change less between pH 6.5 and 7.5, resulting in almost zero slope of $\Delta pK_{on}/\Delta pH$ and $\Delta pK_{off}/\Delta pH$.

**DISCUSSION**

The regulation of the uncoupling activity of UCP is an important factor in the control of thermogenesis. The major players are fatty acids as activators and purine nucleotides as inhibitors of H+ transport by UCP. In addition, the variation of pH influences the nucleotide effect. In BAT cells even in the activated state the concentration of the inhibiting free ATP4− at pH 7.0 may still largely exceed the low $K_c$ and $K_d$ for this nucleotide. Only by increasing the pH above 7.0, the affinity for nucleotides may be sufficiently decreased for generating unliganded UCP (4). A model of the intracellular regulation will be suggested below. Interestingly, this affinity decrease expressed as $\Delta pK_c/\Delta pH$ is much steeper (−2) with nucleoside triphos-
phosphates (GTP and ATP) than (−1) with diphosphates (GDP and ADP) so that with increasing pH, UCP may be rapidly relieved from the most abundant inhibitor ATP4−2.

This pH control was visualized to be due to two H+ -dissociating groups at the nucleotide binding center of UCP, one −CO2H group common for both NDP and NTP and an additional group with a pK of 7.2, e.g. His for NTP only. It was proposed that only the additional positive charge provided by His145 is necessary for binding of NTP4−2 but not for NDP3−2. The −CO2H group pH sensor was previously identified as Glu-190 (6, 7).

It should be noted that out of the four His occurring in UCP, His-214 as well as His-145 and His-147 are conserved among UCP from all sources known so far. His-145 and His-147 have been found by mutagenesis to be involved in H+ translocation.
that His-214 is located in the hydrophilic binding pocket for the His-214 toward DEPC. This suggests, in line with our model, GTP is somewhat lower at pH 6.8 in His-214 mutants and mutants. As a result of the low pH dependence, the affinity for syl-GTP lose their strong pH dependence in the His-214 binding (Fig. 6). Measurements in Mes or Mops buffer (10 mM) at 15 °C was as described under “Experimental Procedures.”

![Graph](image)

**Fig. 6. pH dependence of binding and dissociation rates.** $pK_{on}$ measured by [14C]GTP binding (A) and $pK_{off}$ measured by dansyl-GTP binding (B). Measurements in Mes or Mops buffer (10 mM) at 15 °C was as described under “Experimental Procedures.”

but their replacement did not affect nucleotide binding and its pH dependence (9). Thus only His-214 is left as a candidate for pH sensor of NTP binding. Indeed, here by mutagenesis the involvement of His-214 in nucleotide binding and its pH regulation are shown, although in a somewhat different version than predicted. Both identifications vindicate the original model derived from the pH dependence of nucleotide binding. These results contradict the nondocumented claims by Mordriansky et al. (17) that no histidine and specifically no His-214 is involved in nucleotide binding.

The finding reported here that the His reagent DEPC inhibits binding of GTP but not of GDP and concomitantly that DEPC desensitizes $H^+$ transport against the inhibition by GTP but not by GDP provides evidence for the involvement of a histidine residue in nucleoside triphosphate binding only. The fact that $H^+$ transport is unaffected by DEPC although both His-145 and His-147 have been previously shown to be involved specifically in $H^+$ transport points to a preferred sensitivity of His-214 toward DEPC. This suggests, in line with our model, that His-214 is located in the hydrophilic binding pocket for the $\gamma$-phosphate, where the hydrophilic DEPC can easily enter and due to a high resident time reacts preferably with His-214. In contrast, His-145 and His-147 are either not accessible or are on the surface where DEPC cannot reach a critical concentration.

According to the original model, replacement of His by a neutral group should drastically lower the binding affinity for the nucleoside triphosphates. However, the binding affinity of GTP is found to persist in H214N but is much weaker in H214W. With increasing pH, the affinity decreases much less in H214N and H214W than in wt UCP. The mutations, however, do not change the binding affinity for ADP (which is used here to replace unavailable $[^{14C}]$GDP) showing the same pH dependence as in wt UCP. Similarly, the rates of binding and dissociation as measured with $[^{14C}]$GTP and fluorescent dansyl-GTP lose their strong pH dependence in the His-214 mutants. As a result of the low pH dependence, the affinity for GTP is somewhat lower at pH 6.8 in His-214 mutants and especially in H214W than in wt but is distinctly higher at pH 7.5. This crossover is also clearly seen in the pH dependence of the binding and dissociation rates. The slower dissociation rate of fluorescent (dansyl-GTP) nucleotide observed in H214W versus H214N seems to contradict the lower affinity of His-214 as measured with $[^{14C}]$GTP. Possibly the dansyl derivative of GTP has an additional interaction with the introduced Trp side chain.

The substitution of His-214 by asparagine lowers the stability of UCP upon isolation from mitochondria, and thus the content of intact UCP is about 40% of wt as determined by GTP binding, whereas on substitution by tryptophan, the protein retains its stability. Correspondingly, the capacity for transporting $H^+$ and $Cl^-$ is decreased by about 40% in the H214N and is retained completely in H214W mutant. In relation to the GTP-binding capacity as a measure of intact UCP, the transport activities in H214N are unchanged as compared with wt. Thus, the results with both mutants show that His-214 is not involved in $H^+$ and $Cl^-$ transport. This agrees with the broad evidence that the nucleotide-binding site is not in the translocation channel (18–21).

His-214, located at the c side of the 5th transmembrane helix, seems to be close to the previously established pH sensor Glu-190, located at the c side of the 4th helix. Thus His-214 as well as Glu-190 could be located in the binding pocket for the phosphate moiety of the nucleotide. According to the original model, a His (now His-214) should be primarily in contact with the $\gamma$-phosphate, whereas a $\gamma$-CO$_2$H (now Glu-190) should be at the gate for accessing both nucleoside di- and triphosphate (Fig. 7). Furthermore, HisH$^+$ was assumed to form an ion bond with $\gamma$-phosphate. The marked decrease of the pH dependence of nucleoside triphosphate but not diphosphate binding is in line with the role of His-214 as a pH sensor. However, the retention of NTP binding in His-214 mutants requires a change of our model. At low pH, in H214N, the affinity is only slightly lower than in wt UCP, indicating that HisH$^+$ does not contribute to NTP binding. It now seems that His-214 in the neutral form, abundant at pH > 7 repulses NTP, since the replacement of His by Asn increases affinity at high pH. Our new model, as illustrated in Fig. 7, retained the assumption that His is located at the end of the binding pocket. It shows that the imidazole of the neutral His protrudes into the pocket, thus sterically restraining the binding of triphosphate. On protonization, the HisH$^+$ group is retracted by a background $\gamma$-CO$_2$H group, thus leaving space for the $\gamma$-phosphate. In H214N the smaller Asn side chain does not restrain $\gamma$-phosphate binding, and the pH control is largely lost. In H214W the bulkier side chain may at least partially protrude into the binding pocket as judged from the 9-fold lower binding affinity. In agreement with this model, the His-DEPC derivative, which is unable to accept a $H^+$ and has its hydrophilic extension, would protrude into the binding pocket at any pH and thus block NTP but not NDP binding.

In conclusion, out of the four His only His-214 essentially fulfills the criteria of an additional pH controller specific for NTP binding. Together with the previously identified pH sensor Glu-190, it provides an additional pH regulation of the $H^+$ transport activity of UCP. Both work by changes of electrostatic interaction on $H^+$ binding. Glu-190 as a gatekeeper at the entrance to the phosphate binding pocket, common to NDP and NTP, opens the pocket on protonation, and His-214 makes space for the $\gamma$-phosphate of NTP when being retracted as HisH$^+$ by a negative background charge.

How can the strong pH dependence of NTP binding be incorporated into a consistent picture of the regulation of uncoupling in BAT cells? The major binding nucleotide to UCP can be
assumed to be ATP. In the resting state, at high Mg\textsuperscript{2+} and at pH \textasciitilde7, the free ATP\textsuperscript{4−}:ADP\textsuperscript{3−} ratio is still high despite the lower affinity of ADP\textsuperscript{3−} for Mg\textsuperscript{2+}. Thus, UCP may be well saturated by ATP\textsuperscript{4−}, and evidence for this has been presented by showing that UCP in mitochondria isolated from short term warm-adapted BAT is masked to a large extent by ATP (22). LaNoue et al. (23, 24) made an analysis of the uncoupling regulation in BAT cells and presented impressive arguments in favor of a control by ATP. They estimated that due to the small volume of cytosol in BAT, a small load on ATP would drastically lower the ATP\textsuperscript{4−} concentration. The norepinephrine-induced FA release and ensuing FA activation would generate this ATP load. In addition, lowering the \(\Delta \psi\) on redistribution of ATP into mitochondria contributes to the decrease of cytosolic ATP. The results also suggest that FA somehow modulates the ATP effect on UCP (24). On the other hand, it has been shown by us (25) that there is no competition of FA with nucleotide binding. Therefore we argue that the putative FA effect on ATP inhibition has to be identified with a pH increase to \(\textasciitilde7.2\). After norepinephrine stimulation, mitochondria are flooded with free FA. Their uptake into the matrix can cause a cytosolic pH increase which is becoming more important since the ratio of the cytosol to mitochondrial volume is small. In fact, a 0.8 unit increase of the cytosolic pH relative to mitochondria has been determined on strong load with FA in rat liver perfused with octanoate (26). In addition to lowering the affinity to UCP, the pH increase lowers the ATP\textsuperscript{4−} concentration in favor of ATP-Mg\textsuperscript{2+} formation. Furthermore, as shown by Huang et al. (27), the increase of pH strongly facilitates the otherwise strikingly low dissociation of ATP from UCP. At this point we cannot exclude other mechanisms of pH increase, such as signaling by FA of pH regulation units, e.g. the Na\textsuperscript{+}/H\textsuperscript{+} exchanger, either directly by FA or by AMP which is drastically increased in the cytosol.

This mechanism of the uncoupling regulation in BAT is at variance with that proposed by Nicholls and co-workers (28, 29) who assume that nucleotide binding and dissociation do not play a role in the acute regulation but that in vivo only FA regulates the conductivity of UCP to H\textsuperscript{+}. Nucleotides are assumed to be permanently bound and to shift the conductivity/voltage relation to a nonlinear dependence. Uncoupling occurs with a break potential of 150–170 mV. However, the measurements on isolated BAT indicate that with norepinephrine \(\Delta \psi\) decreases to about 60 mV (24), that is into a range where conductivity of UCP with bound nucleotide would be very low.

It is noteworthy that in the new UCP members, UCP-2 and UCP-3, both Glu-190 and His-214 are conserved, although the homology to UCP-1 is only 59% in UCP-2 and 57% in UCP-3 (30–32). Based on the present results, this indicates that in UCP-2 and UCP-3, nucleotide binding and its pH control follow similar principles as found here for UCP-1. In contrast, His-145 and His-147, previously identified to participate in the H\textsuperscript{+} transport of UCP-1, are not conserved in UCP-2 and UCP-3, indicating a variant mechanism of fatty acid-dependent H\textsuperscript{+} transport.

Acknowledgments—The advice from Dr. Shu-Gui Huang on the use of fluorescent nucleotide derivative and the help of Ilse Prinz for the preparation of mitochondria are gratefully acknowledged.

REFERENCES
1. Nicholls, D. G. (1979) Biochim. Biophys. Acts 549, 1–9
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. Huang, S.-G., and Klingenberg, M. (1995) Biochemistry 34, 349–360
5. Huang, S.-G., and Klingenberg, M. (1995) Biochemistry 34, 145–155
6. Echtay, K. S., Bienengraeber, M., and Klingenberg, M. (1997) Biochemistry 36, 8253–8260
7. Cesareni, G., and Murry, J. A. H. (1987) in Genetic Engineering: Principles and Methods (Sellow, J. K., ed) Vol. 9, pp 135–154, Plenum Publishing Corporation, New York
8. Cesareni, G., and Murry, J. A. H. (1987) in Genetic Engineering: Principles and Methods (Sellow, J. K., ed) Vol. 9, pp 135–154, Plenum Publishing Corporation, New York
9. Klingenberg, M., Winkler, E., and Huang, S.-G. (1995) Methods Enzymol. 260, 369–389
10. Verkman, A. S., Takla, R., Sefton, B., Basbaum, C., and Widdicombe, J. H. (1989) Biochemistry 28, 4240–4244
11. Klingenberg, M., Winkler, E., and Huang, S.-G. (1995) Methods Enzymol. 260, 369–389
12. Miles, E. W. (1977) Methods Enzymol. 47, 431–442
13. Brandsch, M., Brandsch, C., Ganapathy, M. E., and Leibach, F. H. (1997) Biochim. Biophys. Acts 1324, 251–262
14. Lim, J., and Turner, A. J. (1996) FEBS Lett. 381, 188–190
15. Bragg, P. D., and Hou, C. (1996) Eur. J. Biochem. 241, 611–618
16. Mordriansky, M., Murdza-Inglis, D. L., Patel, H. V., Freeman, K. B., and
Garlid, K. D. (1997) *J. Biol. Chem.* **272**, 24759–24762
18. Klingenberg, M. (1984) *Biochem. Soc. Trans.* **12**, 390–393
19. Rial, E., and Nicholls, D. G. (1986) *Eur. J. Biochem.* **161**, 689–694
20. Kopecky, J., Jezek, P., Drahota, Z., and Houstek, J. (1987) *Eur. J. Biochem.* **164**, 687–694
21. Huang, S.-G., and Klingenberg, M. (1996) *Biochemistry* **35**, 7846–54
22. Huang, S.-G., and Klingenberg, M. (1995) *Eur. J. Biochem.* **229**, 718–725
23. LaNeue, K. F., Koch, C. D., and Meditz, R. B. (1982) *J. Biol. Chem.* **257**, 13740–13748
24. LaNeue, K. F., Strzelecki, T., Strzelecka, D., and Koch, C. (1986) *J. Biol. Chem.* **261**, 288–305
25. Winkler, E., and Klingenberg, M. (1994) *J. Biol. Chem.* **269**, 2508–2515
26. Soboll, S., Grundel, S., Schwabe, U., and Scholz, R. (1984) *Eur. J. Biochem.* **141**, 231–236
27. Huang, S.-G., Lin, Q.-S., and Klingenberg, M. (1998) *J. Biol. Chem.* **273**, 859–864
28. Nicholls, D. G., and Locke, R. M. (1984) *Physiol. Rev.* **64**, 1–64
29. Rial, E., Poustie, A., and Nicholls, D. G. (1983) *Eur. J. Biochem.* **137**, 197–203
30. Fleury, C., Neverova, M., Collins, S., Raimbault, S., Champigny, O., Leveymeyrueis, C., Bouillaut, F., Seldin, M. E., Surwit, R. S., Ricquier, D., and Warden, C. H. (1997) *Nat. Genet.* **15**, 269–273
31. Boss, O., Samec, S., Paoloni-Giacobino, A., Rossier, C., Dulloo, A., Seydoux, J., Muzzin, P., and Giacobino, J.-P. (1997) *FEBS Lett.* **408**, 39–41
32. Vidal-Puig, A., Solanes, G., Grujic, D., Flier, J. S., and Lowell, B. B. (1997) *Biochem. Biophys. Res. Commun.* **235**, 79–82