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# Nucleotide Binding to Uncoupling Protein. Mechanism of Control by Protonation†

Martin Klingenberg
*Institut für Physikalische Biochemie der Universität München, Goethestrasse 33, 8000 München 2, FRG*
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ABSTRACT: Nucleotide binding to the isolated uncoupling protein (UCP) from brown adipose tissue of hamster was studied in detail under equilibrium conditions. Besides microequilibrium dialysis and elution chromatography, a rapid anion-exchange procedure was adapted. From the concentration dependence, the $K_D$ and the binding capacity to UCP of ATP, ADP, and GTP and of the ATP analogues 5'-adenylyl imidodiphosphate (AMPPNP) and adenosine 5'-*O*-(3-thiotriphosphate) were determined. Elucidation of the pH dependence of nucleotide binding was the prime topic. From pH 4.6 to 7.5, the $K_D$ varies by almost 2 orders of magnitude, reaching the limits of the equilibrium methods. The $pK_D$ of GTP and ATP decreases from 6.3 to 4.3 with increasing pH. For ADP, the $pK_D$ varies only from 6.0 to 4.8. The intricate course of the pH dependence shows a "break point" of the $pK_D$ around pH 6.3, where the slope ($pK_D$/pH) changes between about -0.2 and -1. Another break point above pH 7.2 produces a $pK_D$/pH = -2 for ATP and GTP only. AMPPNP binding has a lower affinity ($pK_D$ about 5.8-4.1) and a pH dependence slope of -1 with no break. The breaks suggest involvement of the last ionization group ($pK_H \approx 6.7$) of the nucleotide phosphate. This agrees with the absence of a break for AMPPNP and with the shift by $Mg^{2+}$ of the break for ATP to lower pH. The best-fitting model for the pH dependence requires in addition a $H^+$ dissociating group at the binding site of UCP with a $pK_H \approx 4$, dominating the whole pH range. A second group effective above pH 7.0 amplifies the debinding specifically of ATP, not CTP or ADP. Further, the model implies binding of both $NTP^{4-}$ and the protonated $NTPH^{3-}$ or $NDP^{3-}$ and $NDPH^{2-}$ forms, however, with different affinities. On this basis, the relation between the measured overall $K_D$ and the intrinsic $K_D$'s of both nucleotide forms and the various $H^+$ dissociation constants is derived, and the corresponding $pK_D$/pH curves are calculated. A good fit with the data is obtained with a $pK_H = 3.8$ for the UCP center and a $pK_H = 6.8$ for nucleotides and with affinity ratios of 50 for $NTP^{4-}/NTPH^{3-}$ and 100 for $NDP^{3-}/NDPH^{2-}$. The binding of the protonated nucleotide $NTPH^{3-}$ is seen only at a low pH, but with the analogue $AMPPNPH^{3-}$ with $pK_H = 7.6$ it dominates the whole pH range to pH 7.2 with corresponding low affinity. Above pH 7, the dissociation of an additional group at the UCP binding center, probably histidine, is invoked. A binding site model is derived where in the nonbinding state $Glu^-$ or $Asp^-$ by an ion pair blocks $Lys^+$ or $Arg^+$ from binding the anionic nucleotides. In the binding state, $Lys^+$ or $Arg^+$ is set free on protonation of the acidic group. An additional binding regulation above pH 7.0 is interpreted due to protonation of *His*. With these two $H^+$ dissociating groups provided by the nucleotide binding site of UCP and in combination with the $H^+$ dissociation at the nucleotide, an optimized pH profile for the regulation of UCP activity in the brown fat cell is formed.

Nucleotide binding to the uncoupling protein (UCP)[1] of brown adipose tissue has been instrumental in unraveling this key element of the heat production function of the brown adipose tissue. Beginning with the finding of recoupling of respiration by addition of nucleotides to mitochondria (Rafael et al., 1969), followed by photoaffinity labeling of the 32-kDa component (Heaton et al., 1978) and subsequent isolation of UCP (Lin & Klingenberg, 1980), nucleotide binding has been the main assay for defining the protein in the mitochondria

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[1] Abbreviations: UCP, uncoupling protein; AAC, ADP/ATP carrier; TRA, triethanolamine; MES, 2-(*N*-morpholino)ethanesulfonic acid; PIPES, piperazine-*N*,*N*'-bis(2-ethanesulfonic acid); kDa, kilodalton(s); AMPPNP, 5'-adenylyl imidodiphosphate; ATPγS, adenosine 5'-*O*-(3-thiotriphosphate); $AP_5A$, $P^1$,$P^5$-di(adenosine-5') pentaphosphate; EDTA, ethylenediaminetetraacetic acid.

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or in the isolated state. Furthermore, nucleotide–UCP interaction has been an important tool in defining the function of UCP as a $H^+$ conductor both in mitochondria (Nicholls, 1976) and in the reconstituted proteoliposome system (Klingenberg & Winkler, 1985).

A pH influence on nucleotide binding was noted first in mitochondria (Nicholls, 1976). In later work with the isolated protein, a pH dependence was shown to occur only in a range above pH 6.5 whereas below this pH the binding was largely pH independent (Klingenberg & Winkler, 1985). With the isolated UCP available in sufficient amounts in a stabilized form, we embarked on an extensive investigation of the pH dependence of nucleotide binding. Originally, a motivation was the suggestive relationship of $H^+$ involved in nucleotide binding to the $H^+$ interaction site in transport. Thus, information might be obtained on the $H^+$ transport site of this simplest $H^+$ conductor known. In the meantime—as will be discussed below—this regulatory $H^+$ binding site seems to be distinct from the $H^+$ transport site. The primary aim is to elucidate the characteristics of this striking pH dependence which should be important for the regulation of UCP activity. It was an unexpected benefit of these studies that the data could be quantitatively interpreted in terms of a unique ionization mechanism at the binding center which controls nucleotide attachment.

The binding measurements over the wide pH range required several modifications and improvements in the experimental procedures. A new technique, the anion-exchange absorption method, was introduced for facilitating the binding measurements (Klingenberg et al., 1986). Results emerging from this progressing experimental work were already reported previously (Klingenberg, 1984). The present paper represents a full and concise account of the results available so far.

## Materials and Methods

*Buffer Selection.* The incubation buffers had to be carefully selected for covering the pH range in the binding measurements. They had to be tolerated by UCP and to cover, with sufficient buffer capacity, a pH range as wide as possible between pH 4.8 and 7.6. For this purpose, various buffer mixtures were tested. The pH/$H^+$ titration curves were carefully determined. The actual pH at the low incubation temperatures was measured, correcting for the temperature coefficient of the glass electrode.

The following buffers were used:

*Triethanolamine hydrochloride (TRA-HCl)–maleate buffer* was used for the pH range 5.7–7.2. It was prepared from TRA-HCl and maleic acid as a 0.2 M + 0.2 M solution. The pH was adjusted by addition of NaOH at 4 °C.

*Succinate–maleate buffer* was used for the pH range 4.5–6.3; it was prepared from succinic and maleic acids as a 0.2 M + 0.2 M solution by adjustment at 4 °C.

*MES–PIPES buffer* was prepared as a 0.2 M + 0.2 M solution and adjusted at 4 °C.

The radiochemicals [$^{14}$C]ADP, [$^{14}$C]ATP, and [$^{14}$C]GTP were purchased from New England Nuclear Corp., [$^3$H]-AMPPNP was from ICN Chemicals, and [$^{35}$S]ATP$\gamma$S was from Amersham. [$^{32}$P]ATP was prepared from ATP and inorganic $^{32}$P according to Glynn and Chappell (1964).

UCP was prepared according to the procedure of Lin and Klingenberg (1982) without the sucrose gradient centrifugation step. Instead, the eluate from the hydroxyapatite was chromatographed on a G-100 Sephadex column in order to remove the bulk of the phospholipids. The preparation could be stored at 4 °C for 2 weeks, without significant loss of binding capacity.

*Binding Measurements. (A) Elaboration of Measurement Condition.* The method of equilibrium dialysis is theoretically the most accurate and defendable method for measuring the binding of the nucleotides to the solubilized UCP. It is only applicable in an "intermediate range" of ligand affinity, which actually is covered by the present nucleotide–UCP interaction. The greatest disadvantage is the relatively long time required for dialysis equilibration in which the ligand as well as the protein can be changed or inactivated.

A number of experiments were performed to test the stability of the protein binding capacity over the extended incubation time for binding and dialysis. For this purpose, the protein was in the dialysis cells for increasing lengths of time, prior to addition of ATP with an additional dialysis for the actual binding assay. In one example, the binding values were the following: at 4 °C, pH 6.0, with 10 $\mu$M ATP ($\mu$mol/g of protein), 12.5 at 0 h, 11.8 at 3 h, and 10.7 at 6 h; at 25 °C, 8.0 at 0 h and 5.0 at 3 h; at 35 °C, 5.2 at 0 h. We can extrapolate that at 4 °C the binding sites survive the dialysis time of 3 h to about 95%, at 25 °C to 68%, and at 35 °C to 40%. This is further confirmed by comparison of the binding measured with the rapid anion-exchange method and the dialysis method.

The stability of the nucleotides during dialysis was assayed by enzymatic assay of the exposed nucleotides after deproteinization with $HClO_4$ and neutralization with KOH in the two dialysis chambers. Critical for the present case is also the pH influence on the UCP stability. For example, at low pH, where binding is tighter and the amount of free ligand smaller, a small [$^{14}$C]ATP degradation to [$^{14}$C]AMP gives a more serious error than at high pH, where most of the ligands are free.

Particular attention had to be paid to the "ubiquitous" traces of adenylase kinase to which ADP is most sensitive. In a typical experiment (4 °C, 4 h, pH 6.0, 20 $\mu$g of protein/chamber) with 8.5 $\mu$M ADP, 4.2 $\mu$M ADP and 4.3 $\mu$M AMP were found after dialysis. However, on inhibition with 10 $\mu$M $AP_5A$, 8.1 $\mu$M ADP and 0.8 $\mu$M AMP were retained. Therefore, 10 $\mu$M $AP_5A$ and 1 mM EDTA were added throughout to the dialysis buffer. GDP was stable to 95% under the same conditions. Stability assays for ATP gave 90–95% and for GTP 91–97% recovery under a variety of concentrations. ITP was recovered to 80%. The stability is assayed in the presence and absence of protein. At higher pH (7.5), ATP is recovered to 88–92%.

We may conclude that primarily the stability of the UCP limits the time and temperature of dialysis. The stability of the nucleotides can be a major problem. ADP and ATP are more easily degraded than GDP and GTP because of the adenine specificity of adenylate kinase and ATPase. Particularly ADP is labile due to the adenylate kinase reaction. This decomposition can be largely suppressed by adding EDTA and the adenylate kinase inhibitor $AP_5A$.

The amount of protein and nucleotides employed in the binding assay depends on the affinity of UCP in order to measure reasonably accurate ratios of bound/free nucleotides. The relevant mass action relation is

$$[UN] = [U_0] - K_D([UN]/[N])$$

where $[U_0]$ = the concentration of total UCP sites, [U] = the free carrier concentration, [UN] = the carrier–nucleotide complex concentration, [N] = the free nucleotide concentration, $[N_0]$ = the total nucleotide concentration, and $K_D$ = the dissociation constant. Sufficient accuracy for the determination of ratios of bound/free nucleotides ([UN]/[N]) is given only in the range $0.1 < [UN]/[N] < 10$. The concentrations

of $U_0$ and $N_0$ employed should be adjusted so that

$$0.1 < \frac{[U_0] - [UN]}{K_D} = \frac{[U_0] - [N_0] + [N]}{K_D} < 10$$

According to these relations, sets of nucleotide concentrations were chosen for $K_D$ determinations of 0.5–5 $\mu$M for highest affinity and of 5–20 $\mu$M for low affinity. The protein concentration was varied between 40 $\mu$g/mL, corresponding to 0.5 $\mu$M UCP for high affinity at low pH, and 200 $\mu$g/mL ($\approx$2.5 $\mu$M UCP) at high pH.

(*B*) *Measurement by Dialysis.* In order to use as little material as possible and to reduce the equilibration time, a microdialysis cell was used as provided in the "Dianorm" apparatus of Dr. Weder, Zürich, with a chamber volume of only 200 $\mu$L. Twenty cells can be dialyzed in parallel. Stirring is achieved with an air bubble in the rotating cells. The cells can be immersed in a water bath for temperature control. The dialysis membrane is acetylcellulose (Visking) boiled in 0.5% $NaHCO_3$ solution.

A typical assay for determining $K_D$ is as follows. One milliliter of incubation buffer was mixed with 50 $\mu$L of solubilized UCP preparation containing approximately 80 $\mu$g of UCP protein. An aliquot of 200 $\mu$L is pipetted into the right chamber of four assembled microdialysis cells. Two hundred microliters of medium is added into the left chamber, together with increasing nucleotide concentrations. After dialysis for 4 h at 10 °C, the fluid is withdrawn from each chamber with a micropipet, and 100-$\mu$L aliquots herefrom are used for liquid scintillation counting.

The bound nucleotides are calculated from the specific activity and related to the amount of protein employed. These data are evaluated in a "mass action" plot. The ratio of bound/free nucleotides ([UN]/[N]) is obtained from the right and left chambers according to dpm(right) − dpm(left)/dpm(left) = [UN]/[N].

For determining the pH dependency, several sets of four nucleotide concentrations are dialyzed. The protein (UCP) concentration is increased from 40 $\mu$g/mL at pH 5.0–5.8 to 80 $\mu$g/mL at pH 6.0–6.8 and to 150 $\mu$g/mL at pH 7.0–7.6. The added nucleotide concentrations ([$N_0$]) are adjusted to the expected $K_D$ as elucidated above.

(*C*) *Anion-Exchange Method for Binding.* This method takes advantage of the very slow dissociation of the nucleotides from the UCP. It permits a rapid removal of free nucleotides, when the UCP–nucleotide complex passes through a small anion-exchange column before it has time to dissociate during the passage. The pass-through represents the nucleotide bound to UCP. Another advantage is that because of its short duration, it avoids the slow degradation of nucleotides. Also, it can be applied to very small samples.

A small glass capillary column of 2.0 × 60 mm equipped with a sintered glass bottom is filled with 60 mg of wet Dowex 1 × 8, 200–400 mesh, $Cl^-$ form (Fluka AG). The probe contains 10–25 $\mu$g of protein of UCP in only 50 $\mu$L of buffer under addition of the labeled nucleotide (GTP etc.).

The probe is applied on top of the resin with a capillary pipet (e.g., Hamilton type). Then it is immediately chased by squeezing 2 × 200 $\mu$L of $H_2O$ through the column with a plastic pipet. This procedure should take only 1 min. The eluate from the column of about 600 $\mu$L contains all the bound GTP and is used entirely for scintillation counting.

## RESULTS

*ADP/ATP Binding by the Dialysis Method.* A typical concentration dependence for ATP binding as obtained by the dialysis method is given in a mass action plot (Figure 1). Straight lines are fitted through the points which merge approximately at the same maximum binding with the ordinate. The strong pH influence on the binding affinity is clearly evident from the increase of the slopes between pH 6.0 and 7.5. The $K_D$ for ATP is 0.5 × $10^{-6}$ M at pH 6.0 and increases to 3.2 × $10^{-5}$ M at pH 7.5.

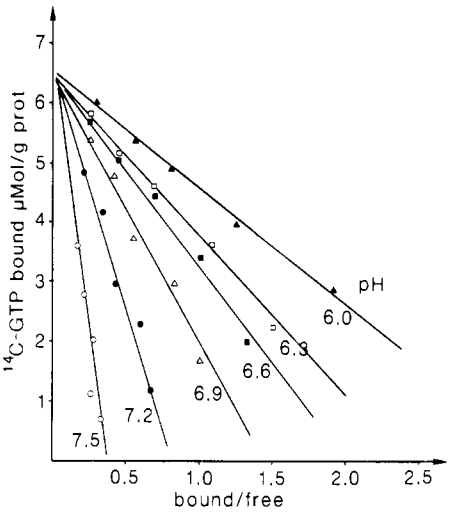


FIGURE 1: Mass action plot of nucleotide binding to UCP at various pHs. Data obtained by the anion-exchange method; 0.23 mg of protein/mL incubated in 10 mM MES–PIPES buffer and 0.5 mM EDTA at 5 °C for 2 h with 2–12 $\mu$M [$^{14}$C]ADP at pH 6.0–6.9 and with 4–25 $\mu$M at pH 7.2–7.5.

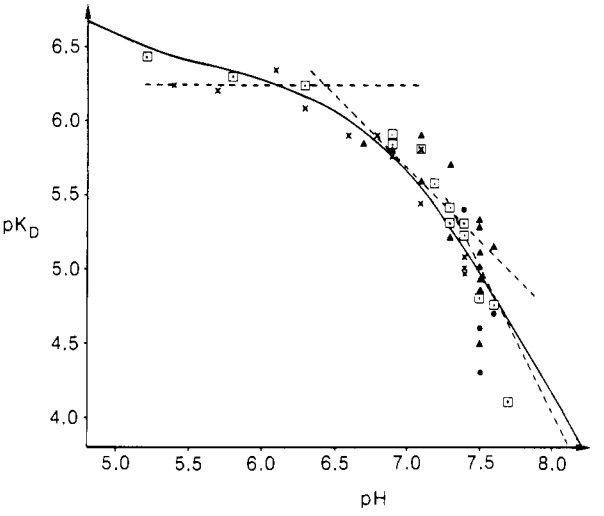


FIGURE 2: Dependence on pH of affinity of ATP binding to UCP. Plot of p$K_D$ versus pH. The line drawn is computed from eq 10a of the text, with $\delta$ = 0.02, $\gamma$ = $10^{-3}$, and $\beta$ = 0.3 (see also Figure 10). Data from four different UCP preparations in six experiments obtained by equilibrium dialysis. The values belonging to each UCP preparation are marked by the same symbols; 0.4–0.65 mg of protein/mL is incubated with 2.5–20 $\mu$M [$^{14}$C]ATP in 25 mM TRA–maleate buffer, 1 mM EDTA, and 10 $\mu$M $AP_5A$ at the pH indicated.

This remarkable pH dependency was the prime focus of extensive measurements by various methods. The p$K_D$ values obtained by the dialysis method for ATP in several experiments are assembled in Figure 2 in a plot of the p$K_D$ vs pH. Only those data are shown which were obtained under conditions where the degradation of ATP is minimized (see Materials and Methods). The pH range covered from pH 5.2 to 7.6, which is limited either by the stability of UCP or by the affinity within the possibilities of the equilibrium measurements. The affinity changes more than 100-fold in this pH range. There seems to be a plateau below pH 6.2 at approximately $K_D$ =

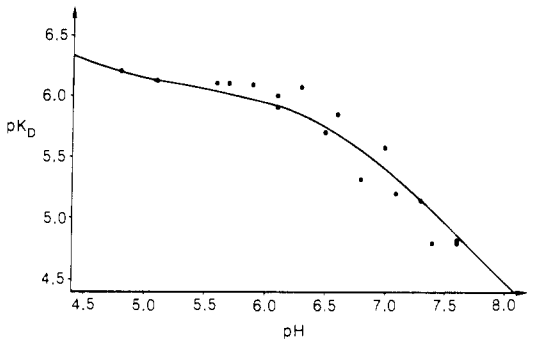


FIGURE 3: Dependence on pH of affinity of ADP binding to UCP. Data from two different UCP preparations in three experiments. The line drawn is computed from eq 9a with $\delta = 0.01$ and $\gamma = 10^{-3}$. Conditions same as for Figure 2.

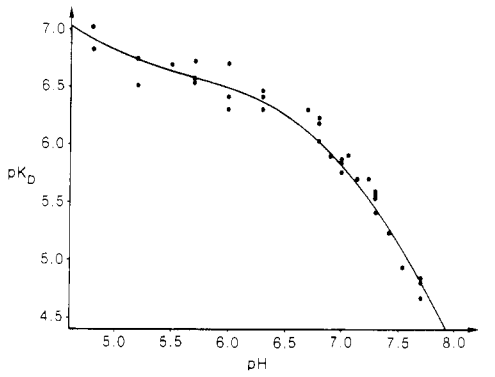


FIGURE 4: Dependence on pH of binding of ATP to UCP as determined by the anion-exchange method. The line drawn is computed from eq 10a with $\delta = 0.02$, $\gamma = 10^{-3}$, and $\beta = 0.3$. Data from three different UCP preparations in four experiments. Incubation of 0.16 mg of protein/mL at pH 4.8–5.5 in 25 mM succinate–maleate buffer, 1 mM EDTA, and 10 $\mu$M $AP_5A$ and of 0.32 mg of protein/mL at pH 5.7–7.7 in 25 mM TRA–maleate buffer and 1 mM EDTA at 0 °C for 40 min.

$0.5 \times 10^{-6}$ M. Above pH 6.2, the $K_D$ increases to nearly $10^{-4}$ M at pH 7.5.

The continuous curve drawn is a computed fit obtained by a detailed analysis given below of the possible contribution of protonation at the nucleotides and at the UCP binding center. Since in the $pK_D$/pH plot often slopes with integral numbers are expected, we have placed corresponding "guide lines" among the measured data, which also point out "break points". The most pronounced break occurs around pH 6.5 with an approximate change of slope from 0 to −1. Another change in the slope from −1 to −2 with a less clearly defined break point is at pH 7.2 which is then followed by a slope of even −3. This precipitous decline of the affinity occurs, however, in the range where the measurements are less accurate.

The question as to how UCP differentiates between nucleoside tri- and diphosphate is of importance for understanding the unique regulatory role of nucleotide binding in the brown adipocyte. We therefore also carefully determined the pH dependency of ADP binding. ADP requires extra protection against the nearly ubiquitous spurious adenylate kinase by addition of $AP_5A$ and AMP (see Materials and Methods). Yet, the extent of decay of about 10% during dialysis remains larger than with ATP. The pH dependency of $K_D$ for ADP binding between pH 4.8 and 7.6 is given in Figure 3 for two different UCP preparations. The pH-induced change of $K_D$ is about 25-fold and distinctly smaller than for ATP. The $pK_D$/pH curve can be fitted nearly with a zero slope below pH 6.3 and a line with a slope of −1 up to pH 7.6. The curve drawn in Figure 3 is computed by parameter fitting, as explained below.

*ATP and GTP Binding by the Anion-Exchange Method.* In view of the various artifacts that can influence the dialysis binding measurements and also in order to verify with another method the striking dependence of the binding on pH, we made elaborate measurements of the binding with the anion-exchange method. Although in principle this violates the equilibrium conditions, this method can be applied under appropriate conditions because of the slow dissociation of the bound nucleotides from UCP. The procedure is more convenient and avoids the long time exposure which may cause the degradation of the protein and nucleotides. It requires, however, a careful selection of the conditions, of buffer, etc. as explained under Materials and Methods. A verification of binding data by two methods was also important in view of the wide range of affinity over nearly 2 orders of magnitude which has to be covered in these measurements.

For binding by the anion-exchange methods, we used primiarly [$\gamma$-$^{32}$P]ATP. It cannot be used in the slow dialysis method because of the hydrolysis by spurious ATPases. The concentration dependence was measured in five different UCP preparations, and a wide range of pHs between 4.9 and 7.7 was covered. The $K_{D'}$, as evaluated from mass action plots, changes about 100-fold in this pH range. As shown in the $pK_D$/pH plot (Figure 4), a similar pH pattern is observed as with equilibrium dialysis, with a small but distinct pH dependence below 6.0 and a stronger decrease at higher pH. The $pK_D$ is slightly higher than in the equilibrium dialysis. This is possibly due to the shorter and correspondingly milder exposure of UCP by this method. The general shape of the $pK_D$/pH relation is very similar in both methods.

The specificity of the binding to UCP has been shown to be limited to purine riboside di- and triphosphates (Lin & Klingenberg, 1982). Thus, GTP and GDP are also good ligands to the UCP. In fact, GDP has been applied in the early binding studies with UCP in mitochondria and also in the solubilized protein (Heaton et al., 1978; Lin & Klingenberg, 1980). Both GDP and GTP have an advantage compared to ADP and ATP in that the degradation by ATPase and adenylate kinase is much slower. For this reason, GTP and GDP have been used primarily as ligands for assaying binding in mitochondria.

The binding of GTP was determined by the anion-exchange method in the dependence on the pH. The results from six experiments with three different UCP preparations are evaluated and shown in Figure 5. Although the affinity of GTP is slightly lower than that of ATP, the course of the pH dependency and the "break point" around pH 6.5 are quite similar. Below pH 6.0, the $K_D$ changes with a smaller slope and a slightly sigmoid shape. Above pH 7.2, the $pK_D$ decreases at a rate $\Delta pK_D/\Delta pH \approx 2$, but distinctly not as steep as that of ATP (Figure 4).

[$^{14}$C]GTP binding has been a cornerstone for comparing various UCP preparations. Because of its stability, it is the preferred ligand for assays of UCP in mitochondria, in sonic particles, and in reconstituted systems. Binding of GDP has been used earlier in brown fat mitochondria and in soluble UCP preparations (Heaton et al., 1978; Lin & Klingenberg, 1982). However, GDP was commercially available only as [$^3$H]GDP which has a 5% contamination of foreign $^3$H label. $^3$H label is relatively easily dissociated from [$^3$H]GDP into water. A pH dependency of [$^3$H]GDP binding to solubilized UCP was previously measured by equilibrium dialysis [see Figure 5 in Lin and Klingenberg (1982)]. Below pH 6.5, a $K_D = 1.5$ $\mu$M was determined. Above pH 6.5, the $pK$ decreases with the slope of $pK_D/pH = -1$.

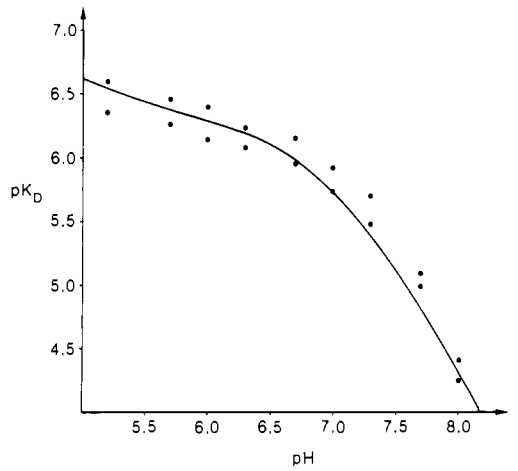


FIGURE 5: Dependence on pH of binding of GTP to UCP as determined by the anion-exchange method. The line drawn is computed from eq 10a with $\delta = 0.02$, $\gamma = 10^{-3}$, and $\beta = 0.3$. Data from three different UCP preparations in three experiments. Incubation of 0.16 mg of protein/mL at pH 5.2–6.5 and of 0.32 mg of protein/mL at pH 6.7–8.0 with 1.25–15 $\mu$M [$^{14}$C]ATP in 25 mM TRA–maleate buffer, 1 mM EDTA, and 12 $\mu$M $AP_5A$ at 0 °C for 40 min.

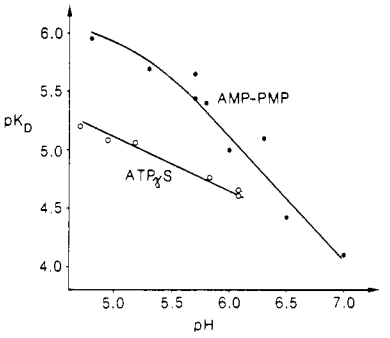


FIGURE 6: Dependence on pH of binding of the ATP analogues AMPPNP and ATP$\gamma$S to UCP. Measurements by the anion-exchange method; 0.32 mg of protein/mL in 25 mM TRA–maleate buffer (pH 5.7–7.0) and 25 mM succinate–maleate buffer (pH 4.8–5.5) with 1.25–12 $\mu$M [$^3$H]AMPPNP and 3–20 $\mu$M [$^{35}$S]ATP$\gamma$S at 0 °C for 45 min.

*Phosphate-Substituted Nucleotide Analogues.* In order to better understand the pH dependence, the binding of nucleotide analogues modified in the phosphate region was also determined. Here, the $H^+$ dissociation constant of the $\gamma$-phosphate is shifted due to the replacement of the oxygen bridge by nitrogen or of the terminal oxygen by sulfur. If the binding–pH relation is found to be changed in accordance with the shifts of $H^+$ dissociation constants, the participation of the $H^+$–nucleotide dissociation is assured. Two analogues of ATP have been used, AMPPNP and ATP$\gamma$S. In AMPPNP, the p$K$ of the last $H^+$ dissociation is more alkaline at p$K$ = 7.8 (Yount et al., 1971), and in ATP$\gamma$S, the p$K$ of the same groups is more acidic at p$K$ = 5.3 (Jaffe & Cohn, 1978). AMPPNP can be obtained in fairly pure $^3$H-labeled form whereas the commercial [$^{35}$S]ATP$\gamma$S is labile and therefore poses difficulties for binding determinations.

As shown in Figure 6, the binding affinity of both of these analogues to UCP is considerably lower than of ATP. For example, at pH 6.0, AMPPNP binds about 12 times weaker and [$^{35}$S]ATP$\gamma$S 20 times weaker. This limits the range of pH measurements for AMPPNP to pH $\leq$7.0. There is a continuous decrease of the affinity from pH 5 to 7, with a slope of p$K_D$/pH $\approx -1$. Below pH 5.2, the curve tends to flatten, probably due to the increasing presence of nonbinding $AMPPNPH_2^{2-}$, since the p$K_H$ for the second $H^+$ dissociation can be expected to be about 4.8. Less reliable are the binding

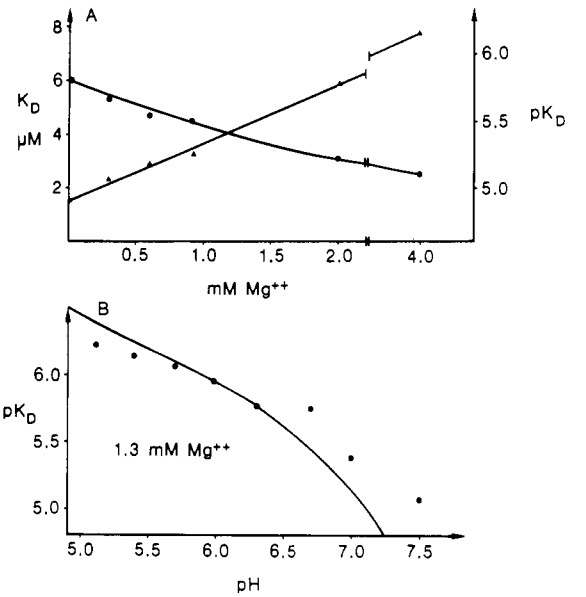


FIGURE 7: Influence of $Mg^{2+}$ concentration on the binding of [$^{14}$C]GTP to UCP. Measurements by the anion-exchange method. The line drawn in (B) is calculated from eq 12 of the Appendix with 1.3 mM $Mg^{2+}$ and the same values for the parameters $\delta$, $\gamma$, and $\beta$ as in Figure 5. (A) Incubation of 0.32 mg of protein/mL in 12.5 mM TRA–maleate buffer with increasing concentrations of $MgSO_4$ and 2–16 $\mu$M [$^{14}$C]GTP for 20 min at 0 °C. (B) Incubation of 0.36 mg of protein/mL at pH 5.1–6.3 in 10 mM PIPES–MES buffer with 0.1 mM EDTA, 1.3 mM $MgSO_4$, and 2–16 $\mu$M [$^{14}$C]GTP for 25 min at 0 °C.

data for [$^{35}$S]ATP$\gamma$S. The p$K_D$ also has a pH dependence but with a weaker negative slope. The fact that both analogues, despite their widely different $H^+$ dissociation constant for the $\gamma$-phosphate, do not exhibit any break point of the pH dependence indicates that the $H^+$ dissociating group at the binding center of the protein has a decisive influence over the $H^+$ dissociating group of the $\gamma$-phosphates. These results are in favor of a participation in ATP binding of protonation at the nucleotide as well as at the UCP, as will be elucidated below.

*Influence of $Mg^{2+}$ and Anions.* Metals, in particular $Mg^{2+}$, are evidently not involved in the binding of the nucleotide to UCP (Lin & Klingenberg, 1982). Therefore, it can be expected that addition of $Mg^{2+}$ decreases the apparent affinity of ATP to UCP because of $Mg^{2+}$–ATP complex formation. Under the influence of $Mg^{2+}$, the pH dependency should change, since $Mg^{2+}$ shifts the apparent p$K_H$ of ATP to a more acidic range because of the higher affinity of $ATP^{4-}$ to $Mg^{2+}$. Thus, the influence of $Mg^{2+}$ should yield information on the contribution to the binding of $H^+$ dissociation at the $\gamma$-phosphate. For these studies, GTP instead of ATP has been used exclusively because of the higher sensitivity of ATP to breakdown by $Mg^{2+}$-activated ATPase and adenylate kinase.

As shown in Figure 7A, $Mg^{2+}$ increases the $K_D$ of GTP binding. As will be derived below, the slope $\Delta K_D/\Delta[Mg^{2+}]$ should be linear and is correlated to the $K_D$ for $Mg^{2+}$ and to the $K_D$ for $H^+$ dissociating from the nucleotide and from the UCP binding site. If $H^+$ dissociation at the nucleotide is involved, $Mg^{2+}$ should shift the pH dependence of the p$K_D$ toward lower pH. Also, the break point in a p$K_D$/pH plot should occur at a lower pH. Results on the pH dependency of nucleotide binding in the presence of $Mg^{2+}$ are evaluated in Figure 7B. The p$K_D$/pH curve is decreased by $Mg^{2+}$, and the slope levels off only in the range below pH 6.0, without a clear sharp break. These results again exclude $Mg^{2+}$–GTP as a ligand. They also support the contention that the pro-

Table I: Summary of Data on the pH Dependency of Nucleotide Binding

| nucleotide | pH range | $pK_D$ | $\Delta pK_D/\Delta pH$ | break pH | method | figure |
|---|---|---|---|---|---|---|
| [$^{14}$C]ATP | 5.2–7.6 | 6.3–4.3 | −1/−2 | 6.5 | dialysis | 2 |
| [$^{32}$P]ATP | 4.8–7.7 | 6.4–4.4 | −1/−2 | 6.2 | ion exchange | 4 |
| [$^{14}$C]ADP | 5.0–7.0 | 6.05–4.8 | −1 | 6.3 | dialysis | 3 |
| [$^{14}$C]GTP | 5.2–8.0 | 6.35–4.3 | −1/−2 | 6.5 | ion exchange | 5 |
| [$^{3}$H]AMPPNP | 4.7–7.0 | 5.9–4.1 | −1 | <5.0 | ion exchange | 6 |
| [$^{35}$S]ATP$\gamma$S | 4.6–6.1 | 5.2–4.6 | −0.5 | | ion exchange | 6 |

Mitochondria

| nucleotide | temp (°C) | pH range | $pK_D$ | $\Delta pK_D/\Delta pH$ | break pH | method |
|---|---|---|---|---|---|---|
| [$^{14}$C]GTP | 0 | 5.1–8.0 | 6.0–4.5 | −0.8 | 6.1 | sedimentation |
| | 22 | 5.1–8.0 | 5.75–4.5 | −1.0 | 6.8 | |

tonation of the nucleotide is involved in the pH dependence. A more detailed analysis will be presented below.

The nucleotide binding is known to be remarkably sensitive to anions (Lin & Klingenberg, 1982). As a representative anion, sulfate competes better than chloride, and it does not have the complication of being a possible transport solute of UCP. As shown by the experiments in Figure 8A, the $pK_D$ becomes more sensitive to increasing sulfate concentration at higher pH. The affinity decreases 10-fold at pH 6.8 and only 3-fold at pH 5.8. Therefore, the $pK_D$/pH relation should be shifted to the more acid range as is indeed shown in Figure 8B. There is no break point down to pH 5.5. This supports the contention that a $H^+$ dissociation group at UCP is involved in the binding. On the other hand, it does not seem to involve the $H^+$ dissociation at the nucleotide. This contradiction is resolved by the observation—to be reported elsewhere—that sulfate strongly decreases the binding rate of the nucleotides. Even within several hours, full equilibration may not have been attained.

A summary of the $pK_D$ values extrapolated from the pH dependence curves in Figures 3–8 is presented in Table I. This table permits a comparison of the binding data between various nucleotides. It cannot describe the intricate pH dependence curves as represented in the figures.

*Evaluation of pH Dependence.* The strong pH dependence is the most striking feature of nucleotide binding to UCP. Its significance is enhanced for two reasons. First, UCP is a $H^+$ translocator, and thus, the pH dependence of the nucleotide binding may reflect $H^+$ binding at the transport site. Second, the pH influence may be instrumental in the intracellular regulation of the UCP activity by controlling the nucleotide binding. In fact, the pH profile should represent the pH control of UCP activation which, by way of its intricate shape, is presumably ideally adapted to the response in the adipocyte. For this reason, a detailed analysis of the pH dependence is of great interest. The following analysis will be restricted to models in which the protonation interferes directly with the binding, and which can therefore be rigidly treated. The possibility that protonation at other sites of the UCP indirectly influences the binding cannot be excluded but will not be considered here for two reasons. First, a reasonable analysis of the pH dependence cannot be performed. Second, if we hold that the pH dependence is of physiological importance, it should have evolved as a specific mechanism at the binding center different from the general protonation of ionizable groups in proteins.

A main question is the identity of the $H^+$ dissociating groups responsible for the pH dependency of the nucleotide binding. An obvious candidate is the $H^+$ dissociation at the terminal phosphate of the nucleotides, e.g.

$$NTPH^{3-} \rightleftharpoons NTP^{4-} + H^+ \qquad NDPH^{2-} \rightleftharpoons NDP^{3-} + H^+$$

These have a $pK$ between 6.5 and 6.9 depending on the ionic strength (Smith & Alberty, 1956). The range would agree

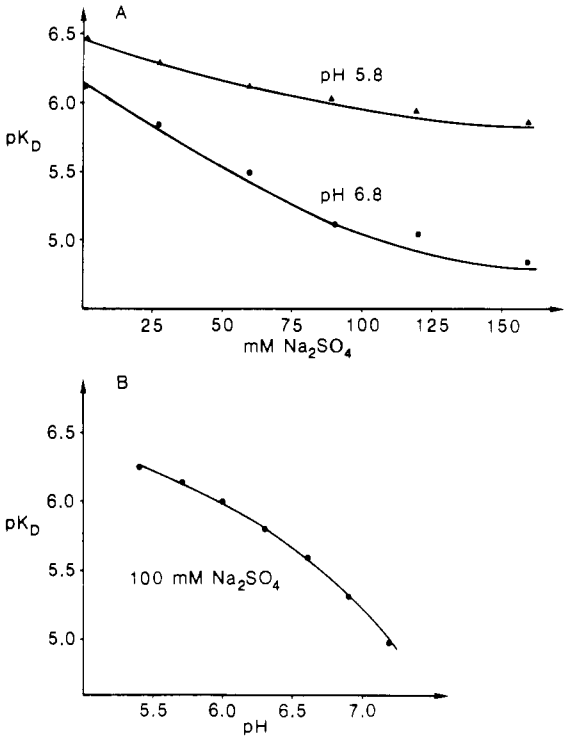


FIGURE 8: Influence of ionic strength (anions) on the binding of [$^{14}$C]GTP to UCP. Measurements by the anion-exchange method and (A) by equilibrium dialysis (at >90–150 mM $Na_2SO_4$). (A) Incubation of 0.21 mg of protein/mL in 12.5 mM TRA–maleate buffer at 0 °C for 2 h with increasing concentrations of $Na_2SO_4$ and 2–16 $\mu$M [$^{14}$C]GTP. (B) Two experiments with 0.16 and 0.32 mg of protein/mL in 25 mM TRA–maleate buffer, ±100 mM $Na_2SO_4$, with 1.2–12 $\mu$M [$^{14}$C]GTP at 0 °C for 40 min.

with the observed pH break point. If we assume that this group is solely responsible for the observed pH dependence, it can be simulated by assuming that only the protonated species $NTPH^{3-}$ or $NDPH^{2-}$ can bind, whereas the fully ionized $NTP^{4-}$ or $NDP^{3-}$ is rejected (model A). In the $pK_D$/pH plot, this model generates a pH independency below and a slope of −1 above the break point.

Principally the same dissociation curve can be obtained by assuming that the relevant $H^+$ dissociation responsible for the break in the $pK_D$/pH curve occurs only at the protein site and that both $NTP^{4-}/NTPH^{3-}$ and $NDP^{3-}/NDPH^{2-}$ bind equally well (model B):

$$U + H^+ \rightleftharpoons UH \qquad U = UCP$$

Only the protonated form of UCP, UH, would bind nucleotide. In this case, a histidine group at the binding center would be a candidate for the protonation with $pK_H < 7.0$.

However, these simple models are unsatisfactory in several respects. There are discrepancies with the measurements; e.g., the measured $pK_D$ still changes in the pH range below the break point although with a much smaller slope than −1. Both

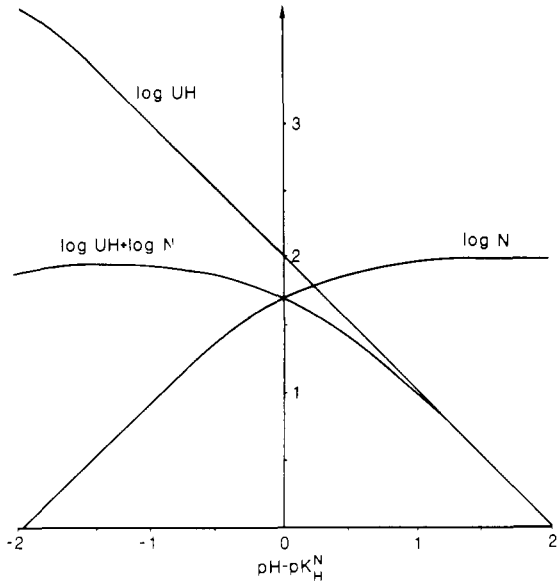


FIGURE 9: pH dependence of nucleotide binding as explained by the superimposition of $H^+$ dissociation both at the nucleotide and at the UCP binding site according to the binding equilibrium [N] + [UH] $\rightleftharpoons$ [UHN] (see text) where N = nucleotide and U = UCP. Plots of log [N], representing the concentration of fully ionized nucleotide $NTP^{4-}$ or $NDP^{3-}$, and of log [UH], representing the concentration of UCP with protonated binding center, vs pH. Adding both curves according to the binding equation gives the pH dependence of $pK_D$.

models do not fit the results with AMPPNP. With a $pK_D$ = 7.6, AMPPNP binding should exhibit no pH dependence according to model A, or it should have the same break point as ATP according to model B. Furthermore, model A is unsatisfactory in that it relies solely on the nucleotide protonation, whereas a fine tuning of the pH control should be developed only by the involvement of the protein. On the other hand, the requirement in model B—that both the fully charged and protonated nucleotides bind equally well—seems improbable.

A more realistic approach incorporates the superimposition of $H^+$ dissociation at the nucleotides and at the protein (model C). In order to illustrate the interplay of $H^+$ dissociation at both binding partners, at first a simplified version is introduced (Figure 9). Again, UCP is assumed to bind only in the protonated form. However, the relevant group at UCP should have a $pK_D$ below the measured pH range. Candidates for these groups are glutamyl with $pK_H$ = 4.2 or aspartyl with $pK_H$ = 3.8. In contrast to model A, the nucleotides bind only in the fully dissociated forms as $NTP^{4-}$ and $NDP^{3-}$. The binding equation is UH + N $\rightleftharpoons$ UH•N, with N representing the fully dissociated nucleotide.

The concentration of the binding species UH ($UCP{\cdot}H^+$) decreases in the log versus pH plot over the whole range at a slope of −1. In contrast, the binding form N (=$NTP^{4-}$ or $NDP^{3-}$) increases with a slope of 1 up to the break point. When both curves are added according to the binding equilibrium, they compensate into a pH independence below and give a slope of −1 above the break point. Thus, by combining the $H^+$ dissociation at the UCP and at the nucleotide, one obtains the same pH dependence as in models A and B. However, it is reasonable that, different from models A and B, the more charged forms $NTP^{4-}$ and $NPD^{3-}$ have a higher affinity than $NTPH^{3-}$ and $NDPH^{2-}$.

Although model C is more attractive with its specifications of nucleotide–protein interaction, it requires further refinement in order to explain the experimental results. At the low-pH branch, one finds a small but distinct increase of the $pK_D$ below the break point, and toward the high-pH end, the $pK_D$ for ATP and GTP decreases more steeply than predicted by this mode. In order to obtain a good fit with the curves, two modifications are introduced which explain the deviations in the lower and upper pH branches. We shall first elucidate the refinement for the lower pH branch.

It is stipulated that in addition to $NTP^{4-}$ and $NDP^{3-}$ (N), also the protonated $NTPH^{3-}$ and $NDPH^{2-}$ (NH) can bind, however, with distinctly lower affinity than the fully ionized forms. The binding reactions are

$$UH + N \overset{K_N}{\rightleftharpoons} UHN \quad \text{and} \quad UH + NH \overset{K_{NH}}{\rightleftharpoons} UHNH$$

The dependence of the total binding on pH, on the two dissociation constants for nucleotide binding, and on the $H^+$ dissociation is given in eq 9 as derived in the Appendix. In order to compare this relation with the measured curves, it was computed for a set of reasonable parameters. For this purpose, eq 9 of the Appendix is rearranged such it has only one $H^+$-dependent variable ($h_N$). For this purpose, $h_U$ is converted into $h_N$ by introducing the ratio of the dissociation constants for nucleotide binding, $\delta = K_N/K_{NH}$, and the ratio of the $H^+$ dissociation constants of UCP and nucleotides, $\gamma = K_H^N/K_H^U$:

$$K_D = K_N \frac{1 + h_N}{1 + \delta h_N}(1 + \gamma/h_N) \qquad (9a)$$

with $h_N = [H^+]/K_H^N$. This equation has been plotted as $pK_D$–$pK_N$ against pH–$pK_H^N$ with various $\gamma$. In Figure 10A, only plots with the ratio $\gamma = 10^{-3.0}$ are shown which permit the best fit with the data. This number is based on the assumption that a glutamyl/aspartyl group at the UCP is responsible for the $H^+$ dissociation ($pK_H^U$ = 3.5–4.2) and for the nucleotide the $pK_H^N$ is 6.4–6.8. The ratio $\delta$ is varied between 0, corresponding to a nonbinding $NTPH^{3-}$, and 1, corresponding to equal binding of $NTPH^{3-}$ and to $NTP^{4-}$. The curves consist of two approximately parallel linear branches which are shifted by approximately log $\delta$, i.e., the difference between $pK_N$ and $pK_{NH}$. The transition between the two branches around the $pK_H^N$ has a sigmoidal slope. The overall decrease is due to the $H^+$ dissociation at the UCP site. The right branch reflects primarily binding of $NTP^{4-}$ with a high intrinsic affinity and the left range, increasingly toward low pH, the binding of $NTPH^{3-}$ with a lower intrinsic affinity. At the left end near the $pK_H^U$, the curves flatten again and in the case $\delta$ = 0 can even decrease.

The lower pH branch of the curve is sensitive to small changes of $\delta$; i.e., it reflects with high sensitivity the binding of $NTPH^{3-}$, even if it may have 2 orders of magnitude lower affinity than $NTP^{4-}$. The measured $pK_D$/pH curve of GTP binding (Figure 5) can be fitted with $\delta$ = 0.02, i.e., with an affinity to UCP of $GTPH^{3-}$ 50-fold lower than that of $GTP^{4-}$. Thus, by allowing also $GTPH^{3-}$ to bind, it is possible to obtain a reasonable fit for the range below pH 6.5.

Also for ATP the measured $pK_D$/pH curves below pH 7 can be quite well simulated by the computed curves, if one fits the low-pH branch with $\delta$ = 0.02 (Figures 2 and 4). However, for ADP and also for GDP [see Figure 5 in Lin and Klingenberg (1982)], the low branch is flatter and comes close to zero slope. It can be fitted with $\delta$ = 0.01 (Figure 3). This means that $GDPH^{2-}$ and $ADPH^{2-}$ are considerably weaker in binding than their protonated triphosphate counterparts $ATPH^{3-}$ and $GDPH^{3-}$.

The pH curve of the AMPPNP binding can now also be explained. In the range above pH 7.0, where binding measurements are possible, it exists mainly as $AMPPNPH^{3-}$ be-

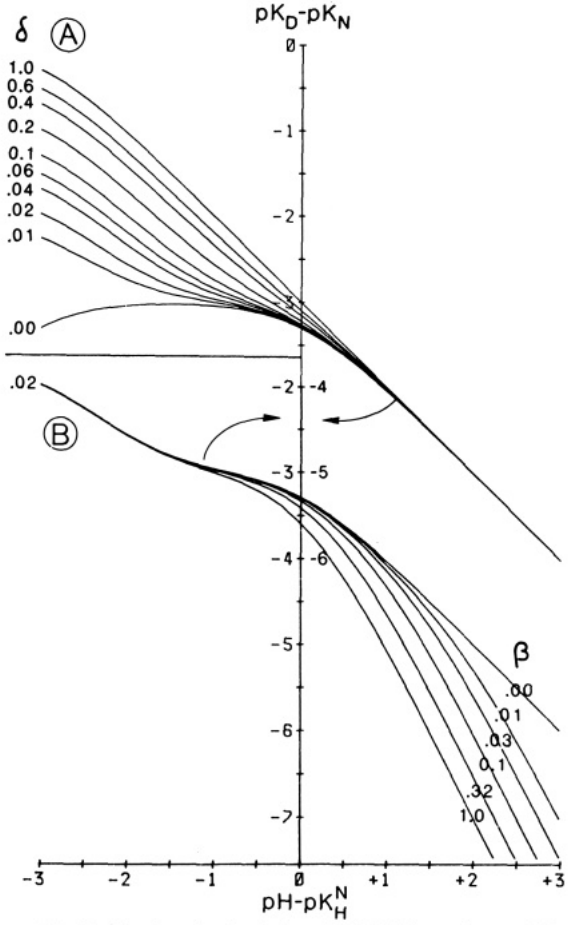


FIGURE 10: Refined analysis of the $pK_D$/pH dependency. The relations are derived in the Appendix. Computations by varying the parameters are explained in the text. For clarity, a wider pH range is chosen than required to fit the actual measurements. The model involves protonation at the UCP binding center and at the terminal phosphate of the nucleotides. (A) For fitting the left branch, the ratio $\delta = K_N/K_{NH}$ is varied over the range 1–0. Best fit for ATP/GTP binding is obtained with $\delta = 0.02$ and for ADP binding with $\delta = 0.01$. The curves are computed with fixed $\gamma = 10^{-3}$, i.e., by assuming a $10^3$ times higher $H^+$ dissociation constant at UCP than at the nucleotide ($pK_H^N - pK_H^U = 3.0$). By varying $\gamma$ (not shown), this value was found to give the best fit. (B) A second protonation at the UCP binding center is introduced for fitting the right branch to the measured data. The dissociation constant is varied with $\beta = K_H^{U'}/K_H^N$. With increasing difference $pK_H^{U'} - pK_H^N$, the "second" break point is shifted to higher pH from the nucleotide dissociation. Other parameters, $\gamma = 10^{-3}$ and $\delta = 0.02$, are fixed and obtained as shown under (A).

cause of its high $pK_H = 7.6$. Therefore, the measured curve actually represents the "left" linear branch of the $pK_D$/pH relation. In fact, an extrapolation of the "left" branch for ATP and GTP into higher pH would fit quite well the measured AMPPNP curve. This would also agree with the 50-fold lower affinity of AMPPNP which is about the same as of the $ATPH^{3-}$ species. In other works, the AMPPNP curve simulates a "pure" $ATPH^{3-}$ binding curve. Also, the absence of a break is in agreement with this interpretation. The bend at the low-pH end can be explained by the emergence of the presumably nonbinding $AMPPNPH_2^{2-}$ species with a probable $pK_H$ around 4.6.

The accelerated decrease in the right branch observed for GTP and ATP binding requires another ramification. The most straightforward way is to invoke an additional $H^+$ dissociation at the active site of UCP. A candidate for a group with a $pK_H$ around 7.2 is histidine. A participation of histidine has been previously implied, based on the loss of GDP binding by photosensitized oxidation of isolated UCP (Lin & Klin-

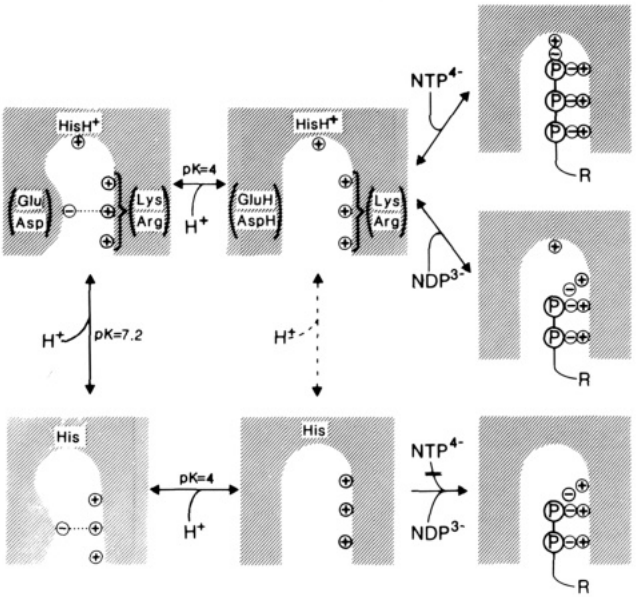


FIGURE 11: Model of the UCP binding center with cationic binding and protonation/deprotonation groups. An acidic group (*Glu* or *Asp*) blocks in the anionic form a cationic group and thus bars the entrance to the binding cleft. On protonation of this group, the cationic group is unmasked, and binding of nucleotides becomes possible. A second protonation/deprotonation, effective above pH 7.0, involves a histidine which provides the fourth cationic charge required for $NTP^{4-}$ binding. On protonation of this group, only $NDP^{3-}$ can bind.

genberg, 1982). This additional deprotonation is superimposed to the putative glutamyl/aspartyl group deprotonation.

The eq 9 for $K_D$ is accordingly extended to eq 10 of the Appendix. For the computations, eq 10 is rewritten. In order to have only one variable ($h_N$) of $H^+$ dependence, $h_U$ and $h_{U'}$ are converted into $h_N$:

$$K_D = K_N \frac{1 + h_N}{1 + \delta h_N}[1 + \gamma/h_N(1 + \beta/h_N)] \quad (10a)$$

The additional parameter $\beta = K_H^{U'}/K_H^N$ is the ratio of the $H^+$ dissociation constants at the second group of UCP and of the nucleotide. The $pK_D$/pH function is computed in Figure 10B by varying $\beta$ in a reasonable range. The slope is highly sensitive to small values of $\beta$ but changes much less with $\beta > 0.31$. The best fit with the GTP binding curve is obtained with $pK_H^{U'} - pK_H^N = 0.31$, i.e., $pK_H^N = 6.8$ and $pK_H^{U'} = 7.1$. Due to the insensitivity against variations in this range, $\beta \approx 0.3$–$0.5$; i.e., $pK_H^{U'} = 7.1$–$7.3$ would fit the measured data.

This additional deprotonation amplifies the $H^+$ sensitivity of the nucleotide debinding and results in a stimulation of UCP activity by small pH changes. It is absent in the pH dependence of binding of ADP and also of GDP which decreases with a slope of only −1. It is reasonable that the less charged and shorter phosphate anhydride region in the diphosphate needs one less positive charge and thus is insensitive to the removal of a positive charge at the binding site (see also the model in Figure 11).

There is an additional precipitous decline of binding affinity above pH 7.4 specifically observed only for ATP. As a result, binding in this pH range seems to differentiate between the guanine and adenine base sections. It may represent a further factor in promoting the interconversion of UCP into the nonbinding form by a minor pH change important for the activation of UCP. However, the low accuracy of equilibrium binding measurements in this range did not permit detailed studies.

The influence of $Mg^{2+}$ on the pH dependence of GTP binding can also be quantitatively assessed and is described

Table II: Intrinsic Dissociation Constants of the Nucleotide Species Binding to UCP[a]

| nucleotide | $pK_N$ | nucleotide | $pK_{NH}$ |
|---|---|---|---|
| $ATP^{4-}$ | 9.05 | $ATPH^{3-}$ | 7.35 |
| $GTP^{4-}$ | 9.0 | $GTPH^{3-}$ | 7.3 |
| $ADP^{3-}$ | 8.6 | $ADPH^{2-}$ | 6.6 |
| | | $AMPPNPH^{3-}$ | 7.25 |

[a] Evaluation according to eq 9, 10, and 11 of the Appendix from the data presented in Figures 3–7, assuming $pK_H^U = 3.8$.

by eq 11 and 12 of the Appendix. In Figure 7B, it is evaluated for $[Mg^{2+}] = 1.3$ mM for the case ($\gamma = 10^{-3}$, $\delta = 0.02$, $\beta = 0.3$) which gives the best fit for the pH dependency of GTP binding. Although the calculated curve shows the same tendency as the data, i.e., a flattening of the "break", it is not able to describe the measured data for so-far unexplained reasons.

In conclusion, with a reasonably good fit between the measured and computed $K_D$ values, the underlying "intrinsic" dissociation constants $K_N$ and $K_{NH}$ can be evaluated. They correspond to the affinity of the protonated UCP binding center to the nucleotide species as defined in eq 4 and 5 of the Appendix. The evaluated dissociation constants are assembled in Table II. The very tight intrinsic binding of the fully ionized nucleotides and the weaker affinity of UCP for the protonated forms are clearly shown.

## Discussion

Nucleotide binding to proteins is widely distributed and has many facets. This is not unexpected because of the fundamental properties and functions of nucleotides. In the vast majority of cases, the nucleotide–protein interaction involves $Mg^{2+}$ which is instrumental in bond splitting. In those rarer cases where nucleotide binding does not follow an enzymatic reaction, no $Mg^{2+}$ is involved. Among these are nucleotides as regulators of enzymatic activity. It is to this category that the nucleotide binding to the uncoupling protein belongs. The nucleotide–protein interaction in the ADP/ATP transporter (AAC) does not involve $Mg^{2+}$ either (Weidemann et al., 1970).

In view of its central significance for the ligand–protein interaction, it seems surprising that relatively few data on equilibrium nucleotide binding are available. Binding studies are often the first step in characterizing the active center. Moreover, the influence of environmental parameters on the binding, such as temperature, pH, ionic strength, etc., is also important for analyzing the influence of these parameters on the function. The relative scarcity of detailed binding studies can be explained by several reasons: the difficulties and problems of equilibrium measurements in general, an unfavorable situation of the $K_D$'s which are either too low or too high, complications due to enzymatic transformation of the nucleotides, or an insufficient appreciation of the importance of equilibrium binding data. The observation that the pH dependence of the nucleotide–protein interaction seems to have received relatively minor attention can also be explained by the fact that the concentrations of Mg–nucleotide complexes, e.g., of $ATPMg^{2-}$ and $GTPMg^{2-}$, are relatively pH independent between pH 6.0 and 8.0, as compared to marked pH dependence of the concentration of $ATP^{4-}$ and $GTP^{4-}$.

*Comparison of Nucleotide Binding to UCP and ADP/ATP Carrier.* Since UCP has a remarkable structural homology with the ADP/ATP carrier (Aquila et al., 1985), a comparison of the nucleotide binding data is significant. The specificity of UCP for nucleotides is considerably lower than that of the AAC (Lin & Klingenberg, 1982; Pfaff & Klingenberg, 1968). In UCP, A and G nucleotides are about equally accepted, whereas in AAC, G nucleotides are excluded. In the phosphate section, the tolerance is very similar; only di- and triphosphates are accepted, but in UCP, the affinity for the triphosphate is slightly higher than for the diphosphate, whereas in AAC the two affinities are reversed. Deoxyribose nucleotides are tolerated by both proteins. Among the four naturally occurring ligands for UCP, ATP is certainly the most important intracellular ligand and regulator because of its high abundance. Still, one finds in the literature the notion that in the adipocytes UCP is liganded with GTP or GDP, although their content is probably 20-fold lower than that of ATP or ADP.

The fact that the affinity of UCP for ATP or ADP is greater than that of AAC seems to contradict the considerably higher specificity of AAC. However, this comparison gave a unique opportunity to illustrate the role of the ligand–protein interaction for the activation energy in transport. It is particularly significant, as the two proteins have a quite similar structure, but in the AAC, the nucleotide activates its own transport, whereas in UCP it inhibits the transport of $H^+$. With the theory of internal binding to catalytic energy transfer in carrier activation these findings have been explained as follows (Klingenberg, 1985). In the AAC, the higher specificity reflects a higher intrinsic binding energy than in the UCP. However, in AAC, a large portion of the intrinsic binding energy is utilized for the conformation change of the active site in preparation of the translocation step, and this results in a lower intrinsic affinity. In UCP, the intrinsic affinity is largely expressed since it is not utilized for an activation energy requirement. In conclusion, the lower intrinsic affinity to UCP may result in a higher extrinsic affinity than in AAC, where the higher intrinsic binding energy is largely compensated by the catalytic energy input. In line with these concepts, nucleotide binding to UCP has been shown to stabilize the protein (Klingenberg, 1985) whereas in the AAC it destabilizes the protein against proteolytic attack by trypsin (Klingenberg et al., 1978).

*Molecular Mechanism of pH Control.* What are the molecular events causing this pH control? The successful analysis of the intricate $pK_D$/pH curves by a quantitative correlation of the $K_D$ to the intrinsic binding constants and the $H^+$ dissociation constants allows a definition of the participating groups in more detail. As stated above, only the direct interference of protonation with the binding will be considered here, although the indirect effect cannot be excluded. The foregoing analysis permitted us to assign one or two ionization groups at the protein in the control of nucleotide binding rather than a generalized effect of protein protonation. On this basis, we shall try to construct a molecular model, which we consider to represent a reasonable interpretation of the data. Here for the sake of simplicity and straightforwardness the protonation is assumed to affect only the residues involved in the binding of the phosphate groups. An influence of protonation of ionizable residues which are involved in the binding of other portions of the nucleotide molecules, such as the purine or ribose, is not considered although conceivable.

The binding center of UCP should contain positive charges for matching the highly anionic nucleotide di- and triphosphates. Lysine and arginine groups usually provide the cationic sites. Three or four positive charges are required for binding, since no $Mg^{2+}$ is involved. For the pH control, an acidic group, *Glu* or *Asp*, is proposed to exist in the binding center which affects binding by ion pair formation to one of the cationic *Lys* or *Arg* groups (Figure 11). Thus, in the deprotonated state of *Glu* or *Asp*, one cationic site is blocked, and consequently, UCP is in the nonbinding state. On pro-

tonation of the anionic group, the cationic site is unmasked and now able to accept a negative charge from the phosphate of the nucleotide. A concomitant local opening at the binding center can further enhance the accessibility.

In addition, effective above pH 7.0, another cationic group involved in the nucleoside binding appears to be dependent on the pH. The $pK_H^{U'}$ = 7.1–7.3 determined for this group suggests that histidine provides the cationic charge. Since this group is essential only for nucleotide triphosphate binding, it can be visualized to represent the fourth cationic charge which is not needed in binding of nucleoside disphosphates ($NDP^{3-}$).

To summarize this model, the binding center is constructed in such a way as to allow a pH-dependent control of nucleotide binding through the protonation of *Glu* or *Asp* and of *His*. The cationic charges necessary for binding are neutralized by one or two salt bridges which are increasingly formed with higher pH. Between pH 5.5 and 6.5, the compensating protonation/deprotonation of the nucleotides makes the affinity fairly pH independent. Above pH 6.8, the pH control by the protein dominates and renders the UCP into the nonbinding state in a rather narrow pH range.

Since UCP is the only $H^+$ translocator known, it represents the simplest example among the wide group of $H^+$ transporting proteins. Therefore, it provides a unique possibility to resolve the molecular mechanism of $H^+$ transport through membrane proteins. Given the $H^+$ dissociating groups at the nucleotide binding center and the fact that UCP is a $H^+$ translocator, one may ask whether the $H^+$ dissociating groups, *Glu* or *His*, are part of the $H^+$ translocating machinery. In the reconstituted system, the $H^+$ transport rate was found to exhibit a rather flat pH dependency between pH 6 and 7.5 (Klingenberg & Winkler, 1986). This would argue against the participation of *Glu*. Furthermore, the $H^+$ translocation rate was observed to be insensitive to diethyl pyrocarbonate, suggesting *His* is not involved. For this reason, we have argued that the nucleotide binding site is not identical with the translocation site and that nucleotide binding controls the activity of the protein indirectly by a conformational change (Klingenberg, 1984). In fact, a transformation of UCP by nucleotide binding into a "tight" conformation has been shown on the basis of the stability against proteolysis, as discussed above. Also, from differential inhibition of binding and $H^+$ transport activity of UCP in mitochondria by chemical modification, a binding of nucleotide not to the translocation site was inferred (unpublished data).

*Role in Regulation.* Since ATP by its abundance is the major intracellular ligand to UCP, the pH control machinery concerns primarily the binding of this species [see also LaNoue et al. (1986)]. With a second $H^+$ accepting group, the pH control in UCP is enhanced specifically for ATP binding. ADP is generally present in much lower concentration than ATP in the cytosol, even if one accounts for the preferred $Mg^{2+}$ complexation of ATP. Moreover, at lower pH, the affinity of UCP for ATP is about 5 times higher than for ADP. However, above pH 7.2, as a result of the less steep decrease with pH, the affinity for ADP can be higher than for ATP. Therefore, the changes in the ratio ATP/ADP under various metabolic situations of the brown adipocytes should be a further factor in controlling the nucleotide binding to UCP. We may conclude that the present data provide a framework by which the intracellular UCP activity is regulated according to the pH and the concentrations of ATP and ADP. It is well possible that additional controlling factors, e.g., fatty acid concentration, may influence the UCP activity (Rial et al., 1983; Cunningham et al., 1986).

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APPENDIX

The aim is to derive the analytical function for the dependence of the measured overall dissociation constant of nucleotide binding to UCP on the dissociation constants of the single ionization species. The contribution of the single equilibria to the overall binding is calculated as a function of the $H^+$ concentration and the $H^+$ dissociation constants. The following assumptions are introduced as elucidated in the text.

Only the protonated form UH (not U) of the binding center of UCP can bind nucleotides. Both the dissociated (N, i.e., $NTP^{4-}$ and $NDP^{3-}$) and protonated (NH, i.e., $NTPH^{3-}$ and $NDPH^{2-}$) nucleotide forms can bind, however, with different affinity. The $H^+$ dissociation equilibrium at the binding center is

$$U + H^+ \rightleftharpoons UH \qquad K_H^U = [U][H^+]/[UH] \qquad (1)$$

$$U = UCP$$

The $H^+$ dissociation at the terminal phosphate of the nucleotides is

$$N + H^+ \rightleftharpoons NH \qquad K_H^N = [N][H^+]/[NH] \qquad (2)$$

$$N = \text{nucleotide}$$

The equilibrium eq 1 and 2 are rewritten

$$[U]h_U = [UH] \qquad [N]h_N = [NH]$$

$$\text{with} \quad [H^+]/K_H^U \equiv h_U \quad \text{and} \quad [H^+]/K_H^N \equiv h_N \qquad (3)$$

The equations for the binding of the nucleotide species N and NH to UCP and the dissociation constants are

$$UH + N \rightleftharpoons UHN \qquad K_N$$

$$UH + NH \rightleftharpoons UHNH \qquad K_{NH}$$

The nucleotide–UCP binding equilibria are

$$[UH][N]/K_N = [UHN] \qquad (4)$$

$$[UH][NH]/K_{NH} = [UHNH] \qquad (5)$$

The total bound nucleotide is

$$[UHN] + [UHNH] = [UH]([N]/K_N + [NH]/K_{NH}) \equiv [B] \qquad (6)$$

For substitution of the total concentration of N and UCP, the conservation equations are introduced:

$$[N_0] = [N] + [NH] + [B]$$

$$[U_0] = [U] + [UH] + [B] \qquad (7)$$

and on substituting eq 3

$$[N_0] = [N](1 + h_N) + [B] \qquad (8a)$$

$$[U_0] = [UH](1 + 1/h_U) + [B] \qquad (8b)$$

Inserting eq 8b into eq 6 gives

$$[B] = \frac{[U_0] - [B]}{1 + 1/h_U}([N]/K_N + [NH]/K_{NH})$$

and inserting eq 3 and 8a gives

$$[B] = ([N_0] - [B])([U_0] - [B])\frac{1/K_N + h_N/K_{NH}}{(1 + h_N)(1 + 1/h_U)}$$

By rearranging this equation, we obtain the overall binding equilibrium and introduce the overall dissociation constant $K_D$:

$$K_D = \frac{([N_0] - [B])([U_0] - [B])}{[B]} = \frac{(1 + h_N)(1 + 1/h_U)}{1/K_N + h_N/K_{NH}} \quad (9)$$

By assuming a second $H^+$ dissociation at the binding center, we differentiate between three ionization species of UCP:

$$U' + H^+ \leftrightarrow U + H^+ \leftrightarrow UH$$

This additional $H^+$ dissociation at the binding center is

$$[U'] + [H^+] = [U] \qquad K_H{}^{U'} = [U'][H^+]/[U]$$

$$[H^+]/K_H{}^{U'} = h_{U'}$$

By retaining the condition that only UH, i.e., neither U nor U', can bind nucleotides, the relation (eq 9) of the overall dissociation constant to the various single constants is extended to

$$K_D = \frac{(1 + h_N)[1 + 1/h_U(1 + 1/h_{U'})]}{1/K_N + h_N/K_{NH}} \quad (10)$$

In the *presence of* $Mg^{2+}$, the relations are as follows. Since no $Mg^{2+}$ is involved in the binding, the sum of the nucleotide species (eq 7) becomes

$$[N_0] = [N] + [NH] + [NMg] + [NHMg] + [B] = [N](1 + h_N + m + h_N m') + [B] \quad (7a)$$

with $m = [Mg^{2+}]/K_{Mg}$ and $m' + [Mg^{2+}]/K_{Mg}'$

$$K_{Mg} = \frac{[N][Mg^{2+}]}{[NMg]} \quad \text{and} \quad K_{Mg}' = \frac{[NH][Mg^{2+}]}{[NHMg]}$$

Equation 10 is extended to

$$K_D = \frac{(1 + h_N + m + h_N m')[1 + 1/h_U(1 + 1/h_{U'})]}{1/K_N + h_N/K_{NH}} \quad (11)$$

Equation 10 can be simplified because for ATP $K_{Mg} = 10^{-4}$ M and $K_{Mg}' = 0.03$ M and with $[Mg^{2+}] < 2$ mM and $h_N m' \ll 1$

$$K_D = \frac{(1 + h_N + m)[1 + 1/h_U(1 + 1/h_{U'})]}{1/K_N + h_N/K_{NH}} \quad (12)$$

**Registry No.** ATP, 56-65-5; ADP, 58-64-0; GTP, 86-01-1; AMPPNP, 25612-73-1; ATPγS, 35094-46-3; L-His, 71-00-1; L-Arg, 74-79-3; L-Lys, 56-87-1; L-Asp, 56-84-8; L-Glu, 56-86-0; Mg, 7439-95-4.

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