Two-Stage Nucleotide Binding Mechanism and Its Implications to H^+ Transport Inhibition of the Uncoupling Protein from Brown Adipose Tissue Mitochondria[†]

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ABSTRACT: The uncoupling protein (UCP) from brown adipose tissue mitochondria is the simplest H^+ translocator known. H^+ transport is regulated by fatty acids as activators and by purine nucleotides as inhibitors. Nucleotide binding again is strongly influenced by the pH [Klingenberg, M. (1988) *Biochemistry 27*, 781−791]. Previously, by using fluorescent 2′-*O*-dansyl (DANS) derivatives of purine nucleotides, a two-stage binding mechanism was unraveled with a slow transition from a loose into a tight conformational state in the isolated UCP [Huang, S.-G., & Klingenberg, M. (1995) *Biochemistry 34*, 349−360]. Whereas with the unsubstituted nucleotides the transition to the tight state is nearly complete, various DANS and DAN (dimethylaminonaphthoyl) nucleotides bind more to the loose state. Here we investigated the relationships between the two-stage nucleotide binding and the inhibition of the H^+ transport activity in reconstituted proteoliposomes. Further, limited tryptic digestion was used as an indicator of conformational change induced by the nucleotide binding in the isolated protein. The inhibition of H^+ transport activity in reconstituted UCP proteoliposomes correlated only with the fraction of tight state of nucleotide binding. Unsubstituted nucleotides (ATP, GTP, and ADP) as well as DANSGTP inhibit fully the H^+ transport, whereas DANSATP and DANSADP inhibit only to about 50%, and DANSAMP is nearly ineffective. Even for the loose conformational state the nucleotide derivatives exhibit considerable affinity. This allows DANSAMP to replace prebound ATP from UCP and relieve the inhibition of H^+ transport by reversing the distribution of UCP from the tight into the loose conformational state. The pH dependence of the fraction of nucleotide binding in the tight state correlates closely with the pH dependence of the degree of H^+ transport inhibition. Titration with DANS nucleotides of UCP incorporated into phospholipid vesicles revealed that over 70% of binding sites had an affinity comparable with that for the isolated UCP while the remaining sites displayed substantially lower affinity, due to nonhomogeneity of the reconstituted system. The sensitivity against trypsin digestion is inversely correlated with the fraction of nucleotide binding in the tight state. Whereas unsubstituted nucleotides and DANSGTP protect strongly against trypsinolysis, DANSATP and DANSADP do only partially, and DANSAMP does not at all. The counteracting influences of the DANS substitution are shown with DANSAMP, which has an affinity comparable to that of DANSATP or DANSADP but cannot form the tight inhibited complex. These data show that nucleotide binding only in the tight state is associated with a strong conformational change, which further causes an inhibition of H^+ transport. In conclusion, UCP can exist in a loose noninhibited and a tight inhibited conformational state. The equilibrium between these two conformations is shifted to the tight state with unsubstituted nucleotides but remains to variable degrees in the loose state with DANS and DAN derivatives. The DANS group hinders progressively the transition to the tight state as the binding affinity of the underlying nucleotide decreases.

The proton gradient across the inner mitochondrial membrane of eukaryotic cells is coupled primarily to ATP synthesis. In brown adipose tissue mitochondria, however, the existence of a highly specialized protein on the inner membrane, called the uncoupling protein (UCP),[1] allows H^+ to return back into the matrix side without passing through the ATP synthase, thus dissipating the electrochemical energy into heat [reviewed in Nicholls and Locke (1984) and Klingenberg (1990)]. Therefore, in these mitochondria, ATP synthesis and substrate oxidation are largely uncoupled. This uncoupling effect depends on the presence of long-chain fatty acids which activate the H^+ transport activity; on the other hand, purine nucleotides inhibit the H^+ transport by UCP. The binding of the inhibitory purine nucleotides has been a key for identifying UCP (Heaton et al., 1978; Lin & Klingenberg, 1980, 1982; Lin et al., 1980). The peculiar pH dependence of nucleotide binding has raised great interest for its potential regulatory role and for elucidating the H^+ dissociating groups at the binding site.

In a previous paper we have characterized the binding of fluorescent 2′-*O*-dansylated (DANS) nucleotides to the purified and soluble UCP (Huang & Klingenberg, 1995a).

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[1] Abbreviations: UCP, uncoupling protein; AAC, ADP/ATP carrier; DAN nucleotide, 3′(2′)-*O*-(5-dimethylaminonaphthalene-1-carboxyl) nucleotide; DANS nucleotide, 2′-*O*-(5-dimethylaminonaphthalene-1-sulfonyl) nucleotide; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); $C_{10}E_5$, *N*-decylpentaoxyethylene.

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The DANS nucleotides show high affinity and characteristic strong pH dependence of binding to UCP similar to the unsubstituted nucleotides (ATP, GTP, ADP). Also, as shown by anion exchange measurements, the binding capacity of UCP for DANSGTP agreed with that for GTP, which was also consistent with the value determined by fluorescence titration. In contrast, the binding capacity for DANSATP determined with the anion exchange method amounted to only about 60% of the value measured by fluorescence titration. This discrepancy was accounted for by assuming that DANSGTP, like the unsubstituted nucleotides, formed primarily a tight complex on binding to UCP, whereas DANSATP formed with UCP 60% tight and 40% loose complex.

In an attempt to understand the relation of the tight and loose complexes to the H^+ transport inhibition, we have investigated the inhibition of H^+ transport activity and the conformational change as monitored by proteolytic sensitivity of UCP under the influence of a broad spectrum of fluorescent nucleotide derivatives. A striking correlation between protection against tryptic digestion, tight complex formation, and inhibition of the H^+ transport activity was found, which was analyzed in terms of a two-stage binding and a correlated H^+ transport inhibition.

MATERIALS AND METHODS

Reagents and Materials. Nucleotides were obtained from Boehringer Mannheim. DANS and DAN nucleotides were prepared according to the procedures of Huang and Klingenberg (1995a) and Klingenberg et al. (1984). Trypsin and chymotrypsin were purchased from E. Merck. Trypsin inhibitor from soybean and chymotrypsin inhibitor phenylmethanesulfonyl fluoride were obtained from Boehringer Mannheim and Serva, respectively. *N*-decylpentaoxyethylene ($C_{10}E_5$) was from Bachem AG (Basel). Triton X-100 was purchased from Sigma. Phosphatidylcholine was isolated from hen egg yolk and purified on Al_2O_3 as described (Klingenberg & Winkler, 1985).

Isolation of UCP. UCP was isolated using Triton X-100 as the solubilizer according to the method of Lin and Klingenberg (1980) but with omission of the further purification step by sucrose density gradient centrifugation. The isolated UCP had essentially one band at 33 kDa as shown by electrophoresis on a 12.5% polyacrylamide gel. Protein concentration was determined according to Lowry et al. (1951) using bovine serum albumin as a standard. The nucleotide binding sites were assessed by [^{14}C]GTP binding using the anion exchange method described in Klingenberg et al. (1986).

Measurements of H^+ Transport Activity. Reconstitution of UCP into phospholipid vesicles was accomplished as described in Winkler and Klingenberg (1992). Briefly, UCP was isolated as described above but by using $C_{10}E_5$ as the detergent and stabilized with phosphatidylcholine after removal of the detergent by treatment with polystyrene beads (Bio-Beads). For reconstitution, the UCP was homogenized with phosphatidylcholine (phospholipid/UCP = 120), an internal medium (100 mM potassium phosphate, pH 7.5), and $C_{10}E_5$ (2.5%). Vesicle formation was accomplished by slow removal of the detergent with polystyrene beads at 4 °C. The external potassium phosphate was removed by passing the vesicles over a G-75 column.

Proton uptake activity was measured on a specially built fast-response pH meter as described in Winkler and Klingenberg (1992) at 10 °C in a standard medium containing 250 mM sucrose, 0.5 mM Hepes, 0.5 mM Pipes, 0.5 mM EDTA, pH 6.8, and 250 μM lauric acid. The final UCP concentration was about 17 μg protein/mL. Valinomycin (1 μM) was added to initiate the K^+ gradient driven H^+ uptake. The capacity was estimated by addition of carbonylcyanide *m*-chlorophenylhydrazone (2 μM). Dilute sulfuric acid was used for calibration of the proton concentration. To assay the inhibition by nucleotides and derivatives, H^+ transport activity was measured after the proteoliposomes were incubated with up to 30 μM nucleotides or derivatives for 10 min. For pH dependence experiments, measurements were conducted in media containing 250 mM sucrose, 0.5 mM Hepes, and 0.5 mM succinate, 0.5 mM EDTA, pH 5.4−7.3.

Limited Trypsinolysis of UCP. Tryptic digestion of the isolated UCP was performed essentially according to the procedure of Eckerskorn and Klingenberg (1987). Triton solubilized UCP (1.0 mg/mL) was digested in 20 mM Mes, pH 6.8, at 30 °C in 0.5-mL Eppendorf cups at UCP/trypsin = 20:1 (w/w). To assay the protection against the digestion, UCP was first incubated with 200 μM nucleotides or the derivatives for 30 min prior to addition of trypsin. Aliquots were removed after digestion for 5, 10, 20, 30, 60 min, and the reaction was stopped by addition of excess trypsin inhibitor. This was followed by electrophoresis analysis on a 12.5% polyacrylamide gel. The extent of digestion was estimated by scanning the gel on a LKB Ultroscan XL scanner (Pharmacia).

Fluorescence Titration. Fluorescence titrations were carried out on a Perkin Elmer fluorescence spectrophotometer (MPF-44A) using 5 × 5 mm quartz cuvettes containing 300 μL of solution. The fluorescence excitation and emission wavelengths were 360 nm and 515 nm, respectively. The slits were set at 6 nm. The fluorescence intensity was corrected for dilution by the titration (<6%), and inner filter effects due to absorbance at 360 nm by the fluorescent nucleotide derivatives according to Lakowicz (1983). Binding of DANS or DAN nucleotides to the reconstituted UCP was measured at 10 °C by titrating UCP in a medium consisting of 250 mM sucrose and 20 mM Mes or Hepes. The unspecific fluorescence was determined in the presence of 1.5 mM ATP (Huang & Klingenberg, 1995a). The micromolar fluorescence ϕ was calculated from the maximum fluorescence enhancement ΔF_{max} divided by the nucleotide binding sites as measured by [^{14}C]GTP binding. The mass action plots constructed from the titration data showed a break, which was interpreted in terms of two populations of nucleotide binding sites. The high/low affinity sites were evaluated from the mass action plot according to the treatment of Weidemann et al. (1970).

Data Analysis. (1) Inhibition of H^+ Transport Activity by Nucleotides. A two-stage reaction scheme is proposed to account for the nucleotide binding and H^+ transport inhibition (see Discussion). We assume that UN and U*N are the concentrations of the loose noninhibited and the tight inhibited UCP−nucleotide complex; K_d = [U][N]/[UN] is the dissociation constant for the loose complex (UN); K_c' = [U*N]/[UN] describes the distribution of the loose and tight complexes. To solve for the concentration of [U*N], we introduce the overall dissociation constant K_D, which mea-

sures both the loose and tight complex, thus,

$$K_D = \frac{[U_o - UN - U^*N][N_o - UN - U^*N]}{[UN + U^*N]}$$

where U_o and N_o are the concentrations of the total UCP and the added nucleotide. Solving for [UN + U*N], we have

$$[UN + U^*N] = \frac{U_o + N_o + K_D - \sqrt{(U_o + N_o + K_D)^2 - 4U_oN_o}}{2}$$

Substituting $[UN] = [U^*N]/K_c'$ into the above equation and solving for [U*N],

$$[U^*N] = \frac{K_c'}{1 + K_c'} \frac{U_o + N_o + K_D - \sqrt{(U_o + N_o + K_D)^2 - 4U_oN_o}}{2}$$

The relative H^+ transport activity is,

$$\frac{V_{H^+}}{V_{H_o^+}} = 1 - \frac{U^*N}{U_o} = 1 - \frac{K_c'}{1 + K_c'} \frac{U_o + N_o + K_D - \sqrt{(U_o + N_o + K_D) - 4U_oN_o}}{2U_o} \quad (1)$$

The relationship between the overall dissociation constant (K_D) and the other constants is derived as follows:

$$K_D = \frac{[Uo - UN - U^*N][N_o - UN - U^*N]}{[UN + U^*N]} = \frac{[U + U^*]N}{UN(1 + K_c')} = \frac{U \cdot N(1 + K_c)}{UN(1 + K_c')} = K_d(1 + K_c)/(1 + K_c')$$

where [U + U*] is the concentration of free UCP at equilibrium and equals [U_o − UN − U*N], N is the concentration of free nucleotide and equals [N_o − UN − U*N], and $K_c = [U^*]/[U]$ describes the distribution of the free UCP in the loose and tight states. Thus the dissociation constant for the loose complex (K_d) can be estimated from the overall dissociation constant K_D and the distribution constant K_c' according to the equation $K_d \approx (1 + K_c')K_D$ since $K_c \ll 1$. The four equilibrium constants are related according to the equation $K_d'/K_c = K_d/K_c'$.

(2) Reactivation of ATP-inhibited H^+ Transport Activity by DANSAMP. We assume that the noninhibitory DANSAMP displaced the fully inhibitory ATP from UCP. The following equations apply under equilibrium conditions,

$$K_D^A = \frac{[U_o - U^*A - UD][A_o - U^*A]}{[U^*A]}; \qquad K_D^D = \frac{[U_o - U^*A - UD][D_o - UD]}{[UD]}$$

where K_D^A and K_D^D are the dissociation constants for ATP and DANSAMP, U_o, A_o, and D_o are the total concentrations of UCP, ATP, and DANSAMP, and U*A and UD are the concentrations of the inhibited UCP−ATP complex and of the loose UCP−DANSAMP complex. Under the experimental conditions, $A_o \gg U^*A$, $[A_o - U^*A] \approx A_o$. Similarly, $[D_o - UD] \approx D_o$. Solving for [U*A], and since H^+ uptake (%) = $100(1 - [U^*A]/[U_o])$, we have

$$H^+ \text{ uptake } (\%) = 100[K_D^A/A_o + K_D^A D_o/(K_D^D A_o)]/[1 + K_D^A/A_o + K_D^A D_o/(K_D^D A_o)] \quad (2)$$

RESULTS

Inhibition of H^+ Transport Activity. To study the inhibitory effects of the DANS nucleotides on the H^+ transport activity of UCP, measurements in vesicles with reconstituted UCP were performed. In earlier studies (Klingenberg & Winkler, 1985; Winkler & Klingenberg, 1992), it was shown that UCP was inserted preferentially in a right-side-out mode in the reconstituted vesicles; therefore, the H^+ transport activity could be largely inhibited by exogenous purine nucleotides; the H^+ transport was driven by a valinomycin induced K^+ diffusion potential and was shown to require fatty acid as an activator. In the following, all measurements were conducted in the presence of 250 μM lauric acid. The initial uptake rate (V) was evaluated from the increase in pH on addition of valinomycin. The H^+ capacity (C_{H^+}) of the vesicles was measured on adding 2 μM carbonylcyanide *m*-chlorophenylhydrazone. The H^+ transport activity was expressed as the first-order rate V/C_{H^+} (min^{-1} mg^{-1} protein) according to Winkler and Klingenberg (1992).

The H^+ uptake activities of UCP at pH 6.8 amounted to 65 min^{-1} mg^{-1} protein, similar to the value reported previously by Winkler and Klingenberg (1992). The concentration dependence of inhibition by the fluorescent nucleotide derivatives and the corresponding unsubstituted nucleotides is shown in Figure 1. The H^+ transport activity decreased hyperbolically with increasing concentrations of the nucleotide or derivatives. Generally, the unsubstituted nucleotides (open symbols) inhibited more strongly than the corresponding DANS nucleotides (filled symbols). ATP, GTP, and ADP inhibited over 80% of the total H^+ transport activity, the residual activity being due to UCP incorporated right-side-in and some unspecific, fatty acid dependent leakage in the vesicles.

The maximum inhibition by the nucleotide derivatives was obtained at near saturating concentrations (30 μM) and was related to that by ATP which was set as 100% (Table 1). Thus DANSGTP inhibited to 88%, whereas DANSATP and DANSADP were only half as strong as ATP. DAN-ATP inhibited only to 21%. DANSAMP (figure not shown) was ineffective since nearly 90% of the activity remained even at saturating DANSAMP concentrations.

For further evaluation, least-squares analysis was performed of the concentration dependence of inhibition according to eq 1 (Materials and Methods). From the fitting

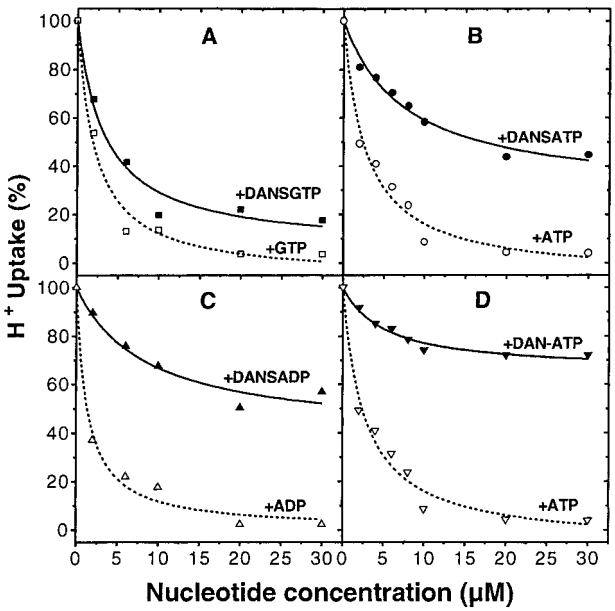


FIGURE 1: Concentration dependence of H^+ transport inhibition by DANS and DAN nucleotides. UCP proteoliposomes containing 17 μg/mL UCP and 2.0 mg/mL phosphatidylcholine were incubated with 250 μM lauric acid and 0−30 μM nucleotides in a medium containing 250 mM sucrose, 0.5 mM Hepes, 0.5 mM Pipes, and 0.5 mM EDTA, pH 6.8, at 10 °C for 10 min. The H^+ transport activity was measured upon addition of 1 μM valinomycin. For comparison inhibition by the corresponding unsubstituted nucleotides was also measured. The H^+ transport activity was 57 min^{-1} mg^{-1} protein and 11 min^{-1} mg^{-1} in the absence and presence of 30 μM ATP. The maximum ATP-inhibited H^+ transport activity (46 min^{-1} mg^{-1} protein) was set as 100%. The theoretical lines were fitted with least-squares analysis according to eq 1 (Materials and Methods). The results are listed in Table 1.

Table 1: Comparison of Inhibition of H^+ Transport Activity of UCP Proteoliposomes by the Unsubstituted Nucleotides and Their Derivatives[a]

	inhibition (%)	K_D[b] (μM)	K_c'[c]	K_d[d] (μM)	K_d/K_c[e]
GTP	100	1.7 ± 0.5	>10^3	>10^3	∼1.7[e]
ATP	100	2.6 ± 0.8	>10^3	>10^3	∼2.6[e]
ADP	100	2.3 ± 0.3	>10^3	>10^3	∼2.3[e]
DANSGTP	88	3.3 ± 0.8	16.2 ± 3.3	56	3.5
DANSATP	57	7.6 ± 1.3	2.6 ± 0.6	28	11
DANSADP	45	8.6 ± 3.3	1.6 ± 0.6	22	14
DANSAMP	9	2.1[f]	∼0.1	∼2.1	21
DAN-ATP	21	5.2 ± 1.0	0.53 ± 0.05	7.9	15

[a] Conditions as given for Figure 1. Least-squares analysis of the data shown in Figure 1 according to eq 1 was performed to estimate the overall dissociation constant (K_D) and the distribution constant (K_c'). The dissociation constant K_d was estimated from $K_d = K_D(1 + K_c')$. [b] Overall K_D, which measures both the loose and tight UCP−nucleotide complexes (see Data Analysis, Materials and Methods). [c] K_c' distribution constant, $K_c' = [U^*N]/[UN]$. [d] K_d, dissociation constant for the loose complex, $K_d = [U]\,[N]/[UN]$. [e] K_d/K_c is calculated from K_d/K_c' since they are equal. For GTP, ATP, and ADP, $K_c' \gg 1$, and $K_c \ll 1$; therefore, $K_d'/K_c = K_D(1 + 1/K_c')/(1 + K_c) \approx K_D$ (see Materials and Methods). [f] From fluorescence titrations (Huang & Klingenberg, 1995a).

we estimated the overall dissociation constant K_D and the distribution constant K_c' for the nucleotides and derivatives. The dissociation constant K_d for the loose complex can be calculated from $K_d = (1 + K_c')K_D$. The results are listed together in Table 1.

As shown in Table 1, the overall dissociation constant K_D for the unsubstituted nucleotides is between 1.3 and 2.6 μM.

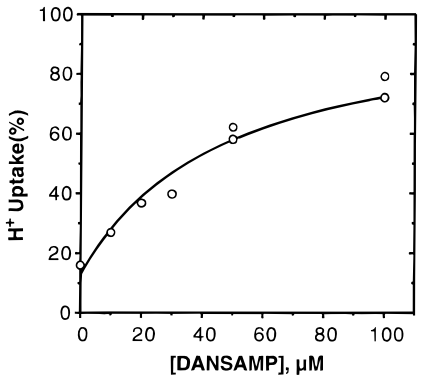


FIGURE 2: Reactivation of ATP inhibited H^+ transport activity of UCP proteoliposomes. UCP proteoliposomes (conditions as for Figure 1) were incubated with 10.5 μM ATP and 0−100 μM DANSAMP in a buffer containing 250 mM sucrose, 0.5 mM Hepes, 0.5 mM Pipes, and 0.5 mM EDTA, pH 7.0. After incubation at 10 °C for 20 min, the H^+ transport activity was measured. The solid line represents a theoretical fitting according to eq 2 with $K_D^A = 1.1 \pm 0.5$ μM, $K_D^D = 4.3 \pm 2.4$ μM.

These results are similar to the dissociation constants reported earlier (Lin & Klingenberg, 1982; Klingenberg, 1988). The K_D for DANSGTP (3.3 μM) is comparable to that for the unsubstituted nucleotides. However, DANSATP, DANSADP, and DAN-ATP display nearly three times higher K_D. The extremely weak inhibition by DANSAMP did not allow an evaluation of the dissociation constant. In Table 1 we gave the K_D value for DANSAMP obtained from fluorescence titrations (Huang & Klingenberg, 1995a). For the unsubstituted nucleotides, we could not accurately obtain the distribution constant (K_c'), since the inhibition was assumed to be 100%. However, a lower limit was estimated to be 10^3 from the fitting procedure. For the nucleotide derivatives, the K_c' decreases in the order unsubstituted nucleotides > DANSGTP > DANSATP > DANSADP > DAN-ATP > DANSAMP (Table 1). The dissociation constant for the loose complex (K_d) decreases in the same order, indicating that the affinity of the nucleotide for the loose conformational state increases accordingly. The ratio K_d'/K_c ($= K_d/K_c'$) should reflect the dissociation constant for the tight state (K_d', see Discussion), which increases from ∼2 for GTP to 21 for DANSAMP, suggesting progressively lower affinity for the tight state.

The rather low inhibition by DANSAMP observed in this work contrasts with the fact that the affinity was only slightly weaker than that of DANSADP and DANSATP (Huang & Klingenberg, 1995a). In order to confirm this, we investigated whether the H^+ transport activity inhibited by nucleotides could be restored by DANSAMP. Thus we measured the H^+ transport activity of the proteoliposomes in the presence of 10.5 μM ATP with further addition of increasing concentrations of up to 100 μM DANSAMP (Figure 2). Under the experimental conditions the inhibition by ATP was 84%. The remaining H^+ transport activity (14%) increased with DANSAMP concentration and reached 80% at 100 μM DANSAMP. The reactivation of H^+ transport can be best explained by the displacement of the prebound inhibitory ATP by the nearly noninhibitory DANSAMP. Indeed displacement by DANSAMP of prebound [^{14}C]ATP was confirmed by anion exchange measurements (Huang, unpublished data). In fact, the reactivation data were best fitted by assuming that DANSAMP does not inhibit the H^+ transport activity but displaces the prebound ATP from the

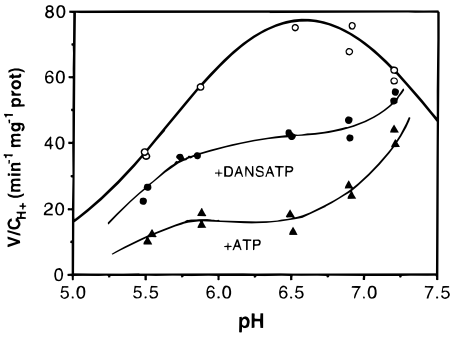


FIGURE 3: pH dependence of H^+ transport activity of UCP proteoliposomes in the presence of DANSATP and ATP. UCP proteoliposomes (conditions as for Figure 1) were incubated with 10 μM DANSATP or ATP in a medium containing 250 mM sucrose, 0.5 mM succinate, 0.5 mM Hepes, and 0.5 mM EDTA, pH 5.4−7.3, at 10 °C. H^+ transport activity was measured as described in Materials and Methods.

binding site. As shown in Figure 2, the solid line was a fit according to eq 2. From this we estimated a dissociation constant K_D of 4.3 ± 2.4 μM for DANSAMP and 1.1 ± 0.5 μM for ATP (pH 7.0). These values are in good agreement with our previous data (Lin & Klingenberg, 1982; Huang & Klingenberg, 1995a).

pH Dependence of H^+ Transport Inhibition. Since nucleotide binding to UCP displays characteristic strong pH dependence (Nicholls, 1976; Klingenberg, 1988; Huang & Klingenberg, 1995a), it is obviously interesting to compare the pH dependency of H^+ transport inhibition with that of nucleotide binding. The inhibition by the fluorescent nucleotide and the corresponding unsubstituted nucleotide was measured at pH ranging from 5.4 to 7.2, after incubation of the UCP proteoliposomes with excess nucleotide. Figure 3 presents an example of the measured H^+ transport activities in the absence and presence of nucleotide at various pH. The H^+ transport activity showed a broad bell shape. At pH < 6.5, the H^+ uptake rate increased with pH, while at pH >6.5 it decreased with pH. In the presence of 10 μM nucleotide, over 95% of the UCP should be saturated with the nucleotide at pH <6.5. The residual H^+ transport activity in the presence of ATP was very low (Figure 3), although it was higher at pH >6.5 due to lower binding. With DANSATP, the shape of pH dependence of the residual activity resembled that of ATP, but at a lower level of inhibition throughout the pH range.

In Figure 4, plots of pH dependence of inhibition were constructed from the measurements shown in Figure 3. A similar pH dependence of inhibition was observed for the DANS nucleotide and the corresponding unsubstituted nucleotide, i.e., the inhibition was flat at pH <6.5 but decreased with pH above 6.5. In the whole pH range, the inhibition by DANSGTP (Figure 4A) was nearly as high as that by GTP or ATP. The inhibition by DANSATP (Figure 4B) and DANSADP (Figure 4C) reached only 40−50%. DANSAMP did not inhibit H^+ transport significantly in the whole pH range studied (figure not shown). DAN-ATP showed a somewhat different pH dependence; the inhibition decreased almost linearly with pH (Figure 4D).

In order to compare the inhibition with the nucleotide binding, we have calculated the pH dependence of binding according to our earlier theoretical model (Klingenberg, 1988; Huang & Klingenberg, 1995a), which are given by the smooth lines in Figure 4. For the unsubstituted nucleotide

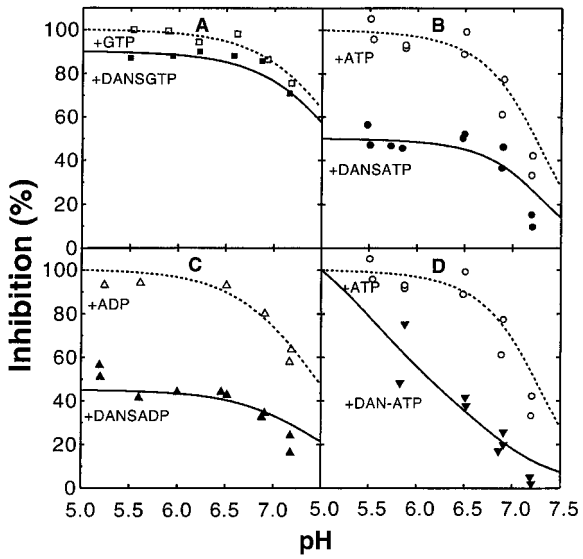


FIGURE 4: pH dependence of H^+ transport inhibition by the DANS and DAN nucleotides. Experimental conditions were as for Figure 3. The inhibition % was evaluated by referring to the maximum ATP inhibition (100%). The smooth lines represent the portion of tight binding calculated according to eq 2, where overall dissociation constants K_D was calculated from the pH dependence models described earlier (Klingenberg, 1988; Huang & Klingenberg, 1995a) and the maximum binding for the unsubstituted nucleotides has been set as 100%. For the unsubstituted nucleotides (dashed lines), we assumed K_c' was so large that $K_c'/(1 + K_c') = 1$; whereas for the fluorescent nucleotide derivatives (solid lines), we assumed a K_c' value of 9.0 for DANSGTP (A), 1.0 for DANSATP (B), 0.69 for DANSADP (C), and 0.2 for DAN-ATP (D).

the inhibition data agree well with the theoretical binding in the whole pH range when the term $K_c'/(1 + K_c')$ in eq 1 (Materials and Methods) was assumed to be 1. Best fits for the nucleotide derivatives were obtained by assuming fractional inhibited tight complex, i.e., by applying varying K_c' values: 9.0 for DANSGTP, 1.0 for DANSATP, and 0.69 for DANSADP. In this way the peculiar inhibition by DAN-ATP (Figure 4D, solid line) can also be explained by the pH-dependent fraction of tight complex (Huang & Klingenberg, 1995a).

Binding of DANS Nucleotides to UCP Proteoliposomes. We have shown previously that the DANS nucleotides bind with strong fluorescence enhancement to isolated UCP (Huang & Klingenberg, 1995a). However, the fluorescence enhancement on binding to UCP reconstituted into liposomes is weaker than to UCP in detergent micelles. Similar to binding to the isolated UCP, the enhanced fluorescence could be reversed by ATP. DANSGTP exhibited the highest fluorescence enhancement, while the fluorescence enhancement for DANSADP and DAN-ATP was about one-third as high.

The results from a typical titration experiment at pH 6.2 are illustrated in Figure 5. As shown in Figure 5A, the unspecific fluorescence (F_{+ATP}) measured by saturating the nucleotide binding sites with excess (1.5 mM) ATP increased linearly with DANSGTP concentration. The specific fluorescence (ΔF) was obtained by subtracting from the total fluorescence (F_{total}) the unspecific fluorescence (Huang & Klingenberg, 1995a). The specific fluorescence ΔF was saturated at low DANSGTP concentration. The titration data for both DANSGTP and DANSATP were evaluated in terms of mass action plots (Figure 5B) constructed according to the procedure of Huang and Klingenberg (1995a). In both

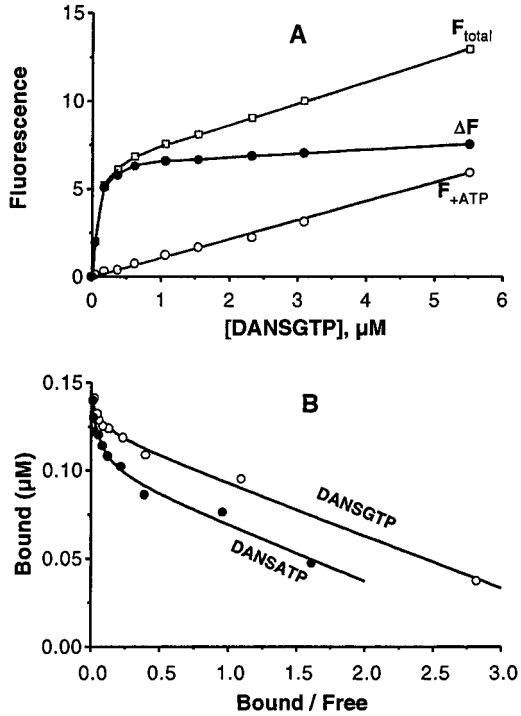


FIGURE 5: Titration of UCP proteoliposomes with fluorescent nucleotide derivatives. (A) Titration curves. UCP proteoliposomes containing 37 μg/mL UCP and 3.7 mg/ml phosphatidylcholine in a medium consisting of 250 mM sucrose and 20 mM Mes, pH 6.2, at 10 °C. F_{total} is the observed fluorescence on addition of DANSGTP, and F_{+ATP} represents the unspecific fluorescence measured in the presence of 1.5 mM ATP. ΔF is the difference ($\Delta F = F_{total} - F_{+ATP}$) and therefore reflects the binding to the UCP sites. (B) Scatchard plot. The concentration of bound nucleotide is evaluated from the specific fluorescence increase ΔF by the formula [bound] $= \Delta F/\phi$ (μM), where ϕ (μM^{-1}) is the micromolar fluorescence yield of bound DANSGTP determined according to Huang and Klingenberg (1995a). The free nucleotide concentration is calculated from mass conservation. The solid lines represent theoretical fittings assuming two binding sites according to the method of Weidemann et al. (1970).

cases a break was observed, which is indicative of loose and tight binding sites in the reconstituted proteoliposomes. For further evaluation we utilized the procedure of Weidemann et al. (1970) for two populations of independent binding sites. From data fitting we obtained the dissociation constants and the corresponding binding sites. For DANSGTP the high-affinity binding amounted to 86% of the total binding sites with a K_D of 0.03 μM, and the lower-affinity sites had a K_D of 1.7 μM. For DANSATP, we estimated a lower proportion of high-affinity sites (71%) with a K_D of 0.03 μM, while the 29% lower-affinity sites had a K_D of 1.8 μM. Titrations at lower pH gave similar shares of the high/low affinity sites despite different values of dissociation constants (Huang, 1994). Titrations at pH >6.5 yielded a more linear mass action plot, corresponding to titrations of the high-affinity sites as the lower-affinity sites had too low affinities to be measured. The dissociation constants of DANSGTP and DANSATP increased to 1.1 and 1.9 μM, respectively, at pH 6.8, which agree well the data obtained with isolated UCP. For DANSADP and DAN-ATP, we obtained quasilinear mass action plots, giving dissociation constants which are comparable to those for the isolated soluble UCP (Huang & Klingenberg, 1995a). Apparently, for these derivatives, the lower-affinity sites were not titrated.

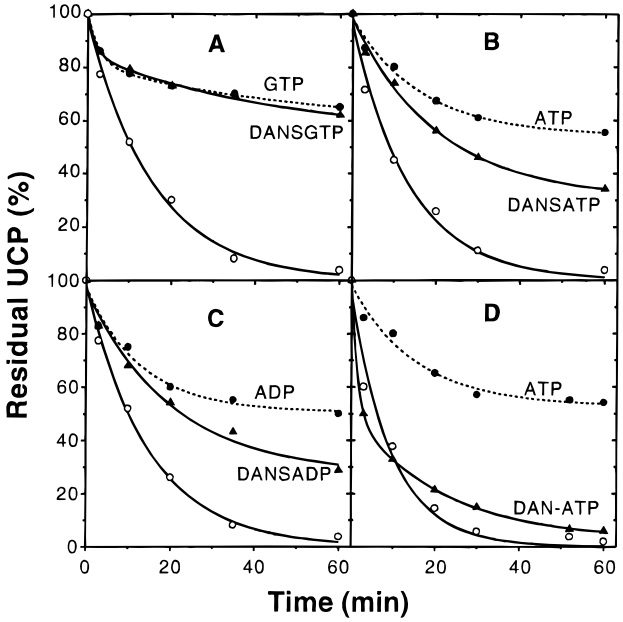


FIGURE 6: Protection against tryptic digestion of UCP by fluorescent nucleotides. Prior to digestion, 1.0 mg/mL UCP had been incubated for 30 min with 200 μM nucleotides in 20 mM Hepes, pH 6.8. Trypsin was added at a ratio of UCP/trysin = 20:1 (w/w), and the whole mixture was incubated at 30 °C. At the time intervals, portions of the reaction mixture were withdrawn and stopped with excess inhibitor, followed by analysis on a 12.5% acrylamide Laemmli gel. The data were evaluated by scanning the gel on an LKB scanner. The results measured in the presence of unsubstituted nucleotide are indicated by solid circles and those in the presence of DANS or DAN nucleotide by upward triangle. Open circles indicate control experiments in the absence of the nucleotide. The smooth lines were fits with a single-exponential decay equation.

Protection Against Limited Proteolysis of UCP. Previously it was shown that the sensitivity of UCP against partial tryptic cleavage was influenced by the binding of nucleotides obviously as a result of a nucleotide induced conformational change in UCP (Eckerskorn & Klingenberg, 1987). Therefore we also examined the effect of the DANS nucleotide derivatives on tryptic digestion. Under the experimental conditions, trypsin cleaves at Lys-292 (Eckerskorn & Klingenberg, 1987; Klingenberg & Appel, 1989), resulting in the formation of a major peptide (T-1) with a molecular mass of 31 kDa and a minor product T-2 (25 kDa). In this work, we observed the formation of the same products, which gave the same pattern on the electrophoresis gel (not shown).

In the absence of nucleotide, tryptic digestion proceeded fast (Figure 6). The UCP was completely digested in 60 min at pH 6.8 and 30 °C with a half-time of about 10 min. To measure the protection against tryptic digestion, an excess of the nucleotides was preincubated with the UCP such that over 96% of the UCP was in the UCP−nucleotide complex form. As shown in Figure 6, the unsubstituted nucleotides and the fluorescent derivatives exhibited varying degrees of protection against the digestion. The unsubstituted nucleotides protected strongly against tryptic digestion; apparently, GTP protected more strongly than ATP and ADP. However, the protection by the DANS and DAN nucleotides was generally weaker as shown by the faster and more extensive digestion even at saturating concentrations. DANSGTP (Figure 6A) protected most strongly, nearly as much as the unsubstituted nucleotides. DANSATP (Figure 6B) and DANSADP (Figure 6C) protected only half as much. DANSAMP (data not shown) and DAN-ATP (Figure 6D)

did not protect significantly. The time course of digestion was fitted with a single-exponential decay equation (smooth lines in Figure 6).

For further evaluation, the capability of protection by the fluorescent nucleotide derivatives was estimated from the digestion data shown in Figure 6. We assumed the protection by ATP as 100% and the protection by the nucleotide derivatives was referred to this value. Thus DANSGTP (100%) protects as strongly as ATP, whereas DANSATP and DANSADP protect by 56% and 50%, respectively. DAN-ATP and DANSAMP do not protect significantly. Although results were not included in the figure, the formation of the digestion product T-1 was opposite to the amount of residual UCP, in line with the capacity of the protection against the digestion.

DISCUSSION

Purine nucleotide binding to the uncoupling protein of brown adipose tissue mitochondria plays a decisive role in the regulation of the H^+ transport and thus the thermogenic activity of UCP. As part of the regulation, UCP exhibits an unusual response to nucleotide binding. Besides a strong and characteristic dependency on pH, it involves clearly differentiated two stages of binding. An initial loose and a subsequent tight UCP−nucleotide complex was derived on the basis of equilibrium binding measurements as well as transient kinetic studies (Huang & Klingenberg, 1995a). The present work further substantiates a two-stage nucleotide binding mechanism and its implication in H^+ transport inhibition, using fluorescent 2′-*O*-dansylated (DANS) nucleotides which have been shown to be valuable probes for studying the binding to the nucleotide site in UCP from brown adipose tissue mitochondria (Huang & Klingenberg, 1995a,b). The two binding stages are associated with a conformational change as monitored by the sensitivity against tryptic digestion. A whole range of distribution between the binding states is seen with the various purine nucleotides and their DANS and DAN derivatives.

Nucleotide-Induced Conformational Change. A nucleotide binding induced conformational change was demonstrated by limited tryptic digestion, confirming an earlier report (Eckerskorn & Klingenberg, 1987). The differential protection against tryptic digestion obtained in this work with a broad spectrum of unsubstituted nucleotides and their DANS and DAN derivatives allows us to assign two conformational states in the UCP, a loose and a tight state. While the free UCP was largely in the loose state which was rapidly cleaved by trypsin, a tightening of the conformation in UCP induced by bound nucleotides prevented the tryptic digestion. The term "tight conformational state" or "tight complex" refers to the latter tight state where tryptic digestion is inhibited.

The trypsinolytic site was shown to be at Lys-292 (Eckerskorn & Klingenberg, 1987). This cleavage site resides within a highly mobile cytosolic C-terminal stretch containing the last 14 amino acids (Klingenberg & Appel, 1989). In fact, the tryptic product T-1 remained dimeric and retained the nucleotide binding affinity (Huang, 1994). On the basis of these findings, we assumed that nucleotide binding induces a conformational tightening in the UCP where at least Lys-292 is shielded from trypsin attack.

Although most of the binding data are collected from measurements with purified soluble UCP (Klingenberg, 1988; Huang & Klingenberg, 1995a), their extrapolation to UCP in proteoliposomes is justified by several binding measurements. The results show that here over 70% of the total binding sites had an affinity comparable to that for the isolated UCP. The portion of lower-affinity sites may have resulted from partial coverage of the binding sites by nearby phospholipid molecules due to nonhomogeneity characteristic of reconstituted systems. With unsubstituted nucleotides, only one binding site was measured by the anion exchange method (Winkler & Klingenberg, 1992, 1994), presumably because the loosely bound nucleotides were retarded by the Dowex. In line with the previous observations (Winkler & Klingenberg, 1992), we interpret this fraction of high-affinity UCP sites as responsible for the measured H^+ transport activity.

Correlation between Nucleotide Binding and H^+ Transport Inhibition. As shown in the results, while the unsubstituted nucleotides (GTP, ATP, ADP) are strong inhibitors of the H^+ transport activity of UCP, the nucleotide derivatives display varying degrees of H^+ transport inhibition. DANSGTP inhibits nearly as strongly as the unsubstituted nucleotides; however, DANSATP and DANSADP inhibited only to about 50% and DANSAMP only to 9%. DAN-ATP had also a weak inhibition (21%) at pH 6.8.

The weak inhibition by DANSAMP permits an impressive demonstration of the relationship between the noninhibitory and inhibitory binding states. DANSAMP shows only slightly weaker binding affinity than DANSADP or DANSATP. Therefore DANSAMP can displace prebound ATP from the UCP and thus reactivate the H^+ transport activity by redistribution of UCP from the inhibited into the largely noninhibited state (Figure 2). The pH dependency of H^+ transport inhibition by the DANS nucleotide is similar to that by the unsubstituted nucleotide, although the degree of inhibition is generally lower. The inhibition data (Figure 4) coincide with the pH dependency of the fraction of nucleotide binding in the tight state calculated according to our previous nucleotide binding model (Klingenberg, 1988; Huang & Klingenberg, 1995a), again indicating that the fraction of tight complex corresponds to the inhibited form.

For illustrating these relations, the fraction of binding in the tight state is plotted against its extent of H^+ transport inhibition as shown in Figure 7. A strong correlation between nucleotide binding in the tight state and H^+ transport inhibition is demonstrated by the straight line with a correlation coefficient of 0.95.

The nucleotides and derivatives can be grouped into three categories according to their degree of H^+ transport inhibition. Unsubstituted nucleotides (ATP, GTP, ADP, GDP) and DANSGTP primarily form tight complexes with UCP and are hence strong inhibitors. DANSATP and DANSADP form only 50% tight complex and are partial inhibitors. DANSAMP and DAN-ATP (pH 6.8) are weak inhibitors due to primary formation of loose complex with UCP. As shown in Table 2, the distribution constant (K_c') evaluated from three different experiments are in fair agreement. Generally the strong inhibitors have $K_c' > 13$, the medium inhibitors K_c' between 0.64 and 2.6, and the weak inhibitors $K_c' < 0.5$.

The Two-Stage Binding Model. The relationship between nucleotide binding, protection against tryptic digestion, and H^+ transport inhibition can be best accommodated by a two-stage nucleotide binding mechanism, in accordance with our previous studies (Huang & Klingenberg, 1995a). A simple

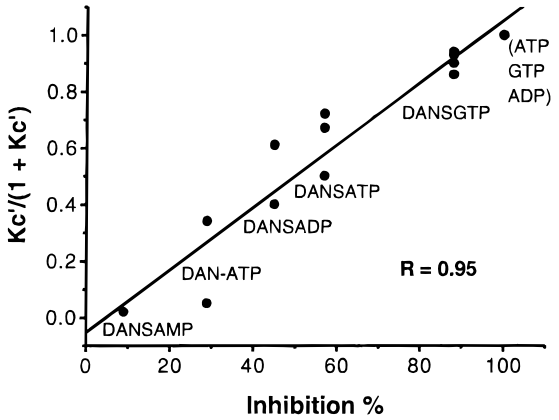


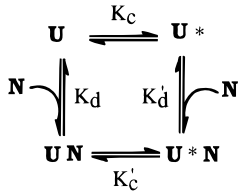
FIGURE 7: Correlation between nucleotide binding in the tight state and H^+ transport inhibition. The inhibition (%) refers to the maximum inhibition (relative to that by ATP; see Text) measured under saturating nucleotide concentrations. K_c' is the distribution constant ($K_c' = [U^*N]/[UN]$) and was estimated from results of anion exchange measurements, protection against tryptic digestion, and H^+ transport inhibition (Table 2). $K_c'/(1 + K_c')$ corresponds to the fraction of the nucleotide binding in the tight state under saturation conditions. A correlation coefficient of $R = 0.95$ was obtained from a linear regression fitting procedure.

Table 2: Comparison of the K_c' Values for the Unsubstituted Nucleotides and Their Derivatives[a]

methods	H^+ transport (pH 6.8, 10 °C)	tryptic digestion (pH 6.8, 30 °C)	anion exchange (pH 6.8, 0 °C)
GTP	$>10^3$	$>10^3$	$>10^3$
ATP	$>10^3$	$>10^3$	$>10^3$
ADP	$>10^3$	9	$>10^3$
DANSGTP	16	13	15
DANSATP	2.6	1.0	2.6
DANSADP	1.6	0.67	nd
DANSAMP	~0	0.02	nd
DAN-ATP	0.53	0.05	nd

[a] The K_c' values were evaluated from (1) the anion exchange measurements (Huang & Klingenberg, 1995a), (2) protection against tryptic digestion, and (3) inhibition of H^+ transport. "nd" stands for not determined.

Scheme 1



scheme similar to our earlier model but without the involvement of H^+ binding is described in Scheme 1 for the nucleotide binding and the associated conformational change.

The uncoupling protein exists in a loose (U) and a tight (U*) conformational state, which are in an equilibrium characterized by the distribution constant K_c (= [U*]/[U]). We further assume that $K_c \ll 1$. The U binds nucleotide or derivative (N) to form an initial loose complex (UN), which transforms through a conformational change into a tight complex (U*N). The tight and loose complexes are in a "slow" equilibrium described by the distribution constant K_c' (= [U*N]/[UN]). The dissociation constants K_d and K_d' relate to the nucleotide binding in the loose and tight conformational states of UCP. Although we could not measure the dissociation constant for the tight complex (K_d') due to the unknown K_c, according to the data in Table 1 the affinity of the nucleotide for the tight conformational state (U*) is found to be approximately inversely proportional to its affinity for the loose state (U).

There are two aspects to interpret the data with the two-stage binding model in thermodynamic and kinetic terminology. First, there is an equilibrium between the two conformational states for which the nucleotides and derivatives show different affinities, and as a result the equilibrium is shifted to varying degrees between the two states. This can be compared with the equilibrium between a relaxed state (R) and tight state (T) reported in effector binding to regulatory enzymes (Stadtman, 1966). A best example is the phosphofructokinase (Uyeda, 1979) where an inhibitor ATP shifted the equilibrium toward the T state, while AMP, ADP, and GDP activate the enzyme by distributing the enzyme in the R state (Shirakihara & Evans, 1987; Lau & Fersht, 1987; Deville-Bonne & Garel, 1992). Similar to the two-stage binding described in the present work, the regulatory site of phosphofructokinase has a specificity for ATP and UTP over CTP while ITP was much less inhibitory (Uyeda & Racker, 1965).

Second, there is a varying kinetic retardation of the transition between the loose and tight states, depending on the type of nucleotide. Although the binding of the unsubstituted nucleotides definitely shifts the equilibrium toward U*N, the transition into the tight state is usually slow, more so with the tigher binding ATP and GTP than with ADP and GDP. The extremely slow transition into the loose state is reflected in the dissociation rates of some DANS nucleotides (Huang & Klingenberg, 1995a). This retardation also enables the segregation of the loose complex from the tight one by rapid passage through an anion exchanger (Huang & Klingenberg, 1995a).

DANSAMP as well as the DAN nucleotides seem to represent the opposite case where the affinity for the loose state of UCP is higher than for the tight state. Striking evidence for this interpretation was provided by simultaneous addition experiments of DAN-ATP and ATP to isolated soluble UCP, where the former fully occupied the UCP at first but was then slowly replaced by ATP (Klingenberg, 1984). This shows that DAN-ATP has a higher affinity for the readily available loose state of UCP than ATP. In the following slow transition into the tight state the affinities for DAN-ATP and ATP are reversed.

The varying binding behavior among the substituted nucleotides was previously explained in terms of sterical reasons based on the different substitutions at the 2′ and 3′ position in the DANS versus DAN nucleotides producing different conformers, making the DAN nucleotide binding energetically unfavorable and therefore not providing enough binding energy for the transition into the tight state.

The molecular reasons for the slow transition reside presumably in important rearrangements which cause a blocking of the translocation channel for H^+ or Cl^-. Nucleotide binding does not directly block the channel, a conclusion drawn earlier by the use of DAN nucleotides. From the H^+ dependence of nucleotide binding, it was concluded that the phosphate moiety binds into a pocket containing positive charges, whose access is possible only when a salt bridge at the entrance to the binding pocket opens on H^+ binding to a Glu/Asp group (Huang & Klingenberg,

1995a). Once the nucleotide is bound, the pocket can rearrange such that the nucleotide is in a more occluded state. Return from this state requires first the dissociation of the H^+ from the occluded Glu/Asp group and is therefore very slow at pH $\leq$7.

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