Fluorescent Nucleotide Derivatives as Specific Probes for the Uncoupling Protein: Thermodynamics and Kinetics of Binding and the Control by pH[†]

Shu-Gui Huang and Martin Klingenberg*

Institute of Physical Biochemistry, University of Munich, Schillerstrasse 44, 80336 Munich, Federal Republic of Germany

Received July 12, 1994; Revised Manuscript Received October 20, 1994[®]

ABSTRACT: Fluorescent 2'-O-dansylated (DANS) purine nucleotides were synthesized. The fluorescence of the nucleotide derivatives is quenched in aqueous solutions but strongly enhanced on binding to the uncoupling protein (UCP) from brown adipose tissue mitochondria. The fluorescence enhancement was 30-, 10-, and 10-fold for DANSGTP, DANSATP, and DANSADP. One mole of DANS nucleotide binds to 1 mol of dimeric UCP. The binding affinity ranges from 10^5 to 10^8 M^{-1}, similar to that of the unsubstituted nucleotides, while dansylation of AMP increases the affinity 50-fold. The pH dependence in the pK_D/pH plots for the DANS nucleotides is basically similar to that for the unsubstituted nucleotides, i.e., for nucleoside diphosphates the slope $\Delta pK_D/\Delta pH < -1$ at pH 5–6.5, $= -1$ at pH > 6.8, and only for triphosphates $= -2$ at pH > 7.2. Two different protonation sites with a $pK_H \approx 4$ (Asp/Glu) and $pK_H \approx 7.2$ (His), only for nucleoside triphosphates, are suggested to be involved in binding. The higher affinity of DANSGTP indicates additional participation in binding of the C-6 oxygen on the guanine. The binding as measured with the anion exchange method agrees with the fluorescence measurement for DANSGTP, whereas for the more loosely binding DANSATP it is 40% lower. This is interpreted in terms of tight/loose UCP–nucleotide complexes, 100% tight complex for DANSGTP (as well GTP or ATP) but 40% loose complex for DANSATP. By measuring the rapid kinetics using the fluorescence signal, the binding rate is found to be fast and fairly constant for the various nucleotides, whereas the dissociation is slow and strongly nucleotide dependent. The rates are pH dependent with $\Delta pk_{on}/\Delta pH = 1$ for all the nucleotides and $\Delta pk_{off}/\Delta pH = -1$ for DANSNTP but more weakly with $\Delta pk_{off}/\Delta pH < -0.5$ for DANSADP and DAN-ATP. The pH dependence of the binding rate corresponds to a protonation at the carboxylate group (Glu/Asp). The high pH dependence of the dissociation rate only for DANSNTP is explained by deprotonation at the HisH$^+$ which is involved only in nucleoside triphosphate binding. This is in line with the very strong pH dependence of nucleoside triphosphate affinity above pH 7 with a $\Delta pK_D/\Delta pH = -2$ as an important regulatory mechanism for the H$^+$ transport activity of UCP. The differences of the DANS nucleotides versus the DAN and unsubstituted nucleotides as well as the nucleoside tri- versus diphosphate are rationalized in a specific H$^+$ dependent regulatory mechanism at the binding site.

The uncoupling protein (UCP)[1] of the inner membrane of brown adipose tissue mitochondria is a key factor responsible for nonshivering thermogenesis in mammals (Smith & Horwitz, 1969; Himms-Hagen, 1976; Flatmark & Pederson, 1975; Nicholls, 1979). The UCP functions by recycling H$^+$ across the inner mitochondrial membrane into the matrix side, thus dissipating the electrochemical energy into heat. Its activity is inhibited by purine nucleotides. This represents an intracellular regulatory mechanism for the heat production in combination with a strong pH dependence of the nucleotide binding affinity. Another important cofactor is the requirement of long-chain free fatty acids for H$^+$ transport [review in Klingenberg (1990)].

After the isolation of UCP in a functional state (Lin & Klingeberg, 1980) and its successful reconstitution into liposomes (Klingenberg & Winkler, 1985; Winkler &

Klingenberg, 1992), some details of the regulation of H$^+$ transport and of the nucleotide binding were unravelled. Particularly the nucleotide binding and its pH dependence were interpreted to be a regulation of the phosphate binding site by protonation of a carboxyl group, such as Asp or Glu, and an additional protonation of His for the nucleoside triphosphate (Klingenberg, 1988).

In order to analyze further the intricate properties and the regulation of nucleotide binding, the use of optical signals such as fluorescence is most desirable. Previously, (dimethylamino)naphthoyl (DAN)-substituted nucleotides were successfully applied to the study of the ADP/ATP carrier (Mayer et al., 1984; Klingenberg et al., 1984, 1985) and also partially for the study of UCP (Klingenberg, 1984). However, DAN-ATP, despite its binding to the UCP, did not inhibit the H$^+$ transport (Eckerskorn & Klingenberg, 1987). Although this finding is of great interest *per se*, these compounds did not mimic the natural ligands. Here we show that dansylated (DANS) nucleotides have the advantage of behaving more like the unsubstituted nucleotides in that they show a similar high affinity and H$^+$ transport inhibition. Similar to the DAN nucleotides, binding of the DANS nucleotides to UCP results in a strong fluorescence enhance-

[1] Abbreviations: UCP, uncoupling protein; AAC, ADP/ATP carrier; DAN nucleotide, 3'(2')-*O*-[5-(dimethylamino)naphthalene-1-carboxyl] nucleotide; DANS nucleotide, 2'-*O*-[5-(dimethylamino)naphthalene-1-sulfonyl] nucleotide; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid.

ment and so provides a most favorable instrument for studying the nucleotide binding to UCP. With the compounds we are able to obtain a detailed understanding of the nucleotide binding to UCP, its thermodynamics and kinetics, its regulation by H^+, and the pertinent groups involved.

MATERIALS AND METHODS

Materials. Dansyl chloride and Triton X-100 were purchased from Sigma. [8-^{14}C]ATP and [2,8-^{3}H]ATP were from New England Nuclear NEN; [8-^{14}C]GTP was from Amersham. Dowex 1 × 8 (200—400 mesh, Cl$^-$ form) was obtained from Fluka.

Preparation of the DANS Nucleotides. The procedure of Wada et al. (1977) was used with several modifications. For a typical preparation, 250 μmol of purine nucleotide was dissolved in 3 mL of water, to which 1500 μmol of dansyl chloride dissolved in 8 mL of acetone was added. Triethylamine was added under stirring to maintain a pH of 10. After reaction for 6 h in the dark, the reaction mixture was neutralized with dilute HCl, evaporated in vacuo (40 °C) to 1 mL, and extracted with 5 × 2 mL of chloroform to remove the unreacted dansyl chloride.

The reaction mixture was then applied to an LH-20 column (1.6 × 80 cm) and eluted with water. Fractions (10 mL) which absorb light at 254 nm and exhibit orange fluorescence at 350 nm illumination were pooled and lyophilized. The resulting solution (1 mL) was purified on a DEAE-32 column (1 × 48 cm) by a gradient of water to 0.6 M triethylammonium bicarbonate, pH 7.2. Fractions corresponding to the dansyl nucleotide were pooled and lyophilized to dryness. The product was obtained as slightly yellowish solids at a yield of 8—20%. Radiolabeled DANS nucleotides, [^{3}H]-DANSATP and [^{14}C]DANSGTP, were prepared in a smaller scale using the same procedure.

Thin layer chromatography was performed on silica plates using *n*-butanol/H$_2$O/acetic acid (10:6:3) as the developing solvent. NMR analysis was performed on a VXR400 NMR instrument (Varian). The concentration of the DANS nucleotide derivatives was calculated from the absorbance at 338 nm using $\epsilon = 3.95 \times 10^3$ M^{-1} cm^{-1} (Watanabe et al., 1981). The DAN nucleotides were synthesized according to Mayer et al. (1984).

Biochemical Preparations. Brown adipose tissue mitochondria were isolated according to the method of Lin and Klingenberg (1980). The UCP was isolated after solubilization with Triton X-100 as described in the same reference but with omission of the further purification by density gradient centrifugation. Bovine heart mitochondria were isolated according to Smith (1967). Mitochondria of *Saccharomyces cerevisiae* and *Neurospora crassa* were isolated according to Knirsch et al. (1989) and Hackenberg et al. (1978). The ADP/ATP carriers were isolated batchwise as described (Klingenberg et al., 1985). Submitochondrial particles were prepared by sonication according to Mayer et al. (1984).

Fluorescence Measurement. Fluorescence spectra were recorded on an MPF-44A fluorescence spectrophotometer (Perkin Elmer). Fluorescence titration of the carriers with the DANS or DAN nucleotide was performed in a 5 × 5 mm cuvette (300 μL) at $\lambda_{exc} = 360$ nm and $\lambda_{em} = 515$ nm. The concentration of the nucleotide derivatives was varied

so that the UCP was saturated from 10 to 90%. The fluorescence was corrected for dilution (<5%). The buffers used were 20 mM Mes or 20 mM Hepes. The specific fluorescence (ΔF) due to binding to the nucleotide binding site of the UCP was obtained by subtracting from the total fluorescence the unspecific fluorescence measured in the presence of 1.5 mM ATP. To evaluate the amount of bound nucleotide, the plot of ΔF versus the nucleotide concentration (D_o) was fitted with the theoretical equation,

$$\Delta F = \Delta F_{max}[U_o + D_o + K_D - \sqrt{(U_o + D_o + K_D)^2 - 4U_oD_o}]/(2U_o) \quad (1)$$

where U_o is the concentration of UCP (μM) and should represent the maximal concentration of bound nucleotide to the UCP. K_D is the dissociation constant (μM). ΔF_{max} is the maximal specific fluorescence increase obtained from the double-reciprocal plot.

To construct the mass action plot, the concentrations of bound (N_b) and free (N_f) nucleotide are evaluated from $N_b = \Delta F/\phi$, $N_f = D_o - N_b = D_o - \Delta F/\phi$, where ϕ is the micromolar fluorescence yield of the DANS nucleotide when 1 μM DANS nucleotide is fully bound to the UCP. The dissociation constants K_D were estimated from the mass action plot according to

$$\Delta F/\phi = U_o - K_D\Delta F/(\phi D_o - \Delta F) \quad (2)$$

The micromolar fluorescence yield (ϕ) was standardized using quinine fluorescence assuming 100 fluorescence units equals the fluorescence of 2.0 μM quinine in 1 N H$_2$SO$_4$.

Transient state fluorescence measurements were carried out on a stopped flow fluorometer constructed in this laboratory. The light path length of the cell was 2 mm. Monochromatic excitation light at 356 nm was obtained from a mercury arc lamp and Bausch & Lomb high-intensity monochromator. Fluorescence emission was measured at 90° to the incident beam through a KV470 filter. The transmitted beam intensity was measured with a photodiode and displayed on a digital VT 105 computer. The fluorescence signal was divided by the incident beam intensity to reduce lamp fluctuations. The mixing of reactants from two columns into the cell was effected pneumatically. The dead time for this instrument was determined to be 3.2 ms. The first-order rate constants were obtained from the linearized equation $\ln[F_{max}/(F_{max} - F)] = k/t$, where F_{max} is the maximal fluorescence ($t = \infty$), F the fluorescence at time t, and k the first-order rate-constant.

Binding Experiment. The binding of [^{14}C]GTP, [^{14}C]-DANSGTP, [^{14}C]ATP, and [^{3}H]DANSATP to UCP was measured using rapid anion exchange chromatography (Klingenberg et al., 1986). This method is based on the finding that the nucleotides bound to UCP dissociate very slowly from the protein. Therefore by a rapid passage through a small column of an anion exchanger (Dowex), only the free nucleotide is adsorbed. However, as will be shown below, the part of loosely bound DANS nucleotide derivatives can also be trapped by the anion exchanger and thus be segregated from the tightly bound nucleotide.

Chemical Modification. A number of amino acid reagents were used to modify the UCP. After the chemical modification was completed, the residual UCP was measured by

DANSGTP binding in a reference buffer (20 mM Hepes, pH 6.8) at room temperature.

RESULTS

Physical Properties of DANS Nucleotides. The dansyl nucleotides exhibit two absorption peaks at 254 and 338 nm, with $A_{254}/A_{338} = 5.8$ for DANSATP and 6.6 for DANSGTP. In aqueous solution, all the dansyl derivatives have a fluorescence emission maximum at 574 nm when excited at 350 nm. The fluorescence of DANS nucleotides is substantially quenched, e.g., the quantum yield of DANSATP at pH 6.8 is 0.007, based on the quantum yield of quinine (0.70) in 1 N H_2SO_4 (Scott et al., 1970). The quantum yield is enhanced 19-fold in 90% dioxane—water solution, and the emission maximum is shifted to 540 nm.

In the proton NMR spectra (not shown) for DANSATP and DANSGTP, the chemical shifts of the ribose protons were $\delta_{H2'} = 5.40$ ppm and $\delta_{H3'} = 4.85$ ppm for DANSATP and 5.70 and 4.83 ppm for DANSGTP, respectively. Thus substitution of the dansyl ring has occurred at the 2'-O-position of the ribose ring, as evidenced by the chemical shift difference of $\delta_{H2'} - \delta_{H3'} = 0.55$ ppm for DANSATP and 0.87 ppm for DANSGTP; the chemical shift differences for ATP and GTP are 0.10 and 0.20 ppm, respectively. Furthermore, according to the number of aromatic protons, only monosubstitution by the DANS group has occurred. The NMR spectra of DAN-ATP indicate that it consists of a mixture of both 3'-O-DAN-ATP and 2'-O-DAN-ATP in a ratio of 3:2.

Interaction of DANS and DAN Nucleotides with the AAC. The DAN nucleotides bind to the AAC from bovine heart mitochondria with strong fluorescence enhancement, in agreement with the previous observation (Klingenberg et al., 1985). However, they do not interact with the AAC from mitochondria of *S. cerevisiae* and *N. crassa*. The DANS nucleotides produce no fluorescence change when added to the AAC from any source tested as demonstrated at three levels, i.e., the mitochondria, their submitochondrial particles, and the isolated Triton-solubilized carriers. In order to determine whether these ligands bind to the AAC but in a nonfluorescent mode, binding of [³H]DANSATP was measured using the gel filtration method. The results show that DANSATP indeed does not bind to the AAC.

Interaction of DANS Nucleotides with Isolated UCP. General Characteristics. The DANS nucleotides give a strong fluorescence enhancement upon binding to the isolated UCP. Figure 1 shows the typical fluorescence spectra of UCP in the absence and presence of DANSGTP. In the presence of DANSGTP, the fluorescence level is high with an emission maximum at 517 nm when excited at 360 nm. This blue shift of the emission maximum ($\Delta\lambda = -57$ nm) suggests binding of the dansyl group to a hydrophobic region in accordance with the observed fluorescence enhancement and blue shift in organic solvents. Upon addition of ATP, the fluorescence decreases because ATP completes for the nucleotide binding site. With large excess ATP, all the bound DANS nucleotide should be removed and the remaining fluorescence is due to the unbound DANS nucleotide. The excitation peak at 287 nm reflects fluorescence energy transfer between the DANS group and tryptophane (two per monomer). All DANS nucleotides show similar spectra. This applies also to the DAN nucleotides, which are employed for comparison throughout the studies.
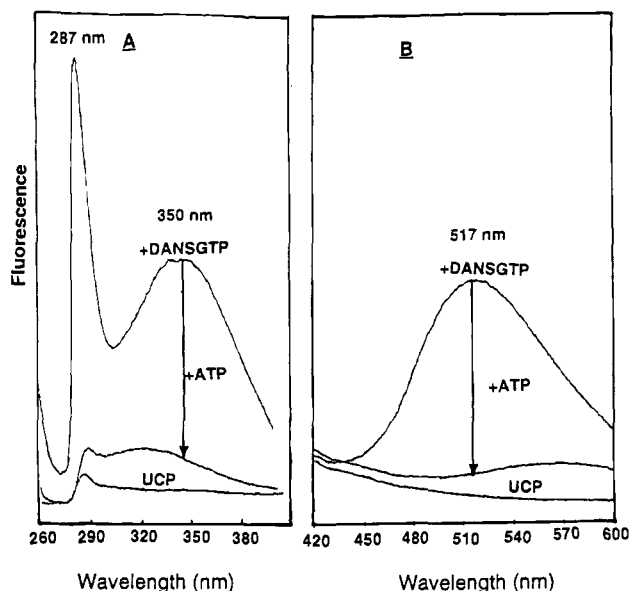

FIGURE 1: Fluorescence spectra of DANSGTP bound to the isolated UCP. Fluorescence excitation spectra (A) and emission spectra (B) were recorded of 40 μg/mL UCP in the absence and presence of 5 μM DANSGTP at 10 °C in 20 mM Mes buffer, pH 6.8. After adding 1.5 mM ATP, the fluorescence decreased. The excitation spectra were recorded at $\lambda_{em} = 515$ nm and the emission spectra at $\lambda_{exc} = 360$ nm.

Figure 2 records the fluorescence response observed at 360 nm excitation and 515 nm emission. Upon addition of DANSGTP, a rapid increase of fluorescence followed by a slower phase is observed. On addition of DAN nucleotides and DANSADP, the fluorescence signals attain the maxima more rapidly than DANSNTP (N = G or A). Similar differences are observed in the dissociation rates of the fluorescence decrease induced by the addition of excess ATP. Whereas the fluorescence of DANSNTP decreases slowly, the dissociation rate is larger for the DAN nucleotides and DANSADP. The difference (ΔF) between the total fluorescence and the fluorescence in the presence of large excess ATP is used as a measure of binding to UCP.

Binding Stoichiometry. Typical titration curves using the calculated ΔF are shown in Figure 3A. To evaluate the DANS nucleotide binding stoichiometry, fluorescence titrations are performed and the specific fluorescence increase is fitted according to eq 1 (Materials and Methods). The U_o's for DANSGTP and DANSATP are found to be close and amount to 2.6 ± 0.4 μM or 11.4 ± 2.0 μmol/g of protein.

For comparison, the binding of [¹⁴C]GTP to the same UCP preparation was also measured using the anion exchange method; this gives the binding capacity $B_{max} = 11.2$ μmol/g of protein. The two values agree well. They indicate that DANSGTP and DANSATP, like the nucleotide (Lin & Klingenberg, 1980), bind at a molar ratio more at one rather than two per UCP dimer. These results also show that for evaluating the fluorescence titration the [¹⁴C]GTP binding capacity can be used. For this purpose, the micromolar fluorescence ϕ (μM⁻¹) is introduced as a relative measure of the specific fluorescence yield in the UCP—DANS nucleotide complex. The ϕ is obtained by relating the ΔF_{max} to the micromolar concentration of the UCP binding sites measured by [¹⁴C]GTP or [¹⁴C]ATP. Thus the concentration of bound nucleotide is related to the specific fluorescence increased by $N_b = \Delta F/\phi$ (μM).
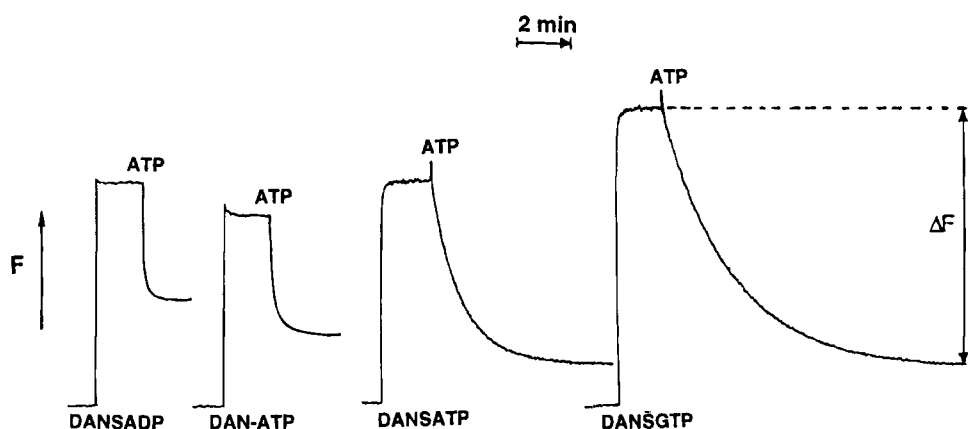
Fluorescence 350-525 nm

FIGURE 2: Fluorescence response of DANS and DAN nucleotides binding to the isolated UCP. Fluorescent nucleotide derivatives (10 μM) were added to a solution of 40 μg/mL UCP in 20 mM Mes buffer, pH 6.6, at 10 °C. After fluorescence equilibrium, 1.5 mM ATP was added to displace the fluorescent ligands from the binding sites. Fluorescence was observed at $\lambda_{exc} = 350$ nm and $\lambda_{em} = 525$ nm.

Table 1:   Summary of Dissociation Constants (K_D) and Micromolar Fluorescence $(\phi)^a$

	DANS				DAN		
	GTP	ATP	ADP	AMP	ATP	ADP	AMP
K_D (μM)	0.30	0.80	1.4	2.1	2.1	1.5	1.9
ϕ (μM^{-1})	80	25	24		20	21	

^{a} The K_D and ϕ values were evaluated from fluorescence titration of 40 μg/mL UCP at pH 6.8 and 10 °C. The micromolar fluorescence ϕ has been corrected with quinine fluorescence as described in Materials and Methods.
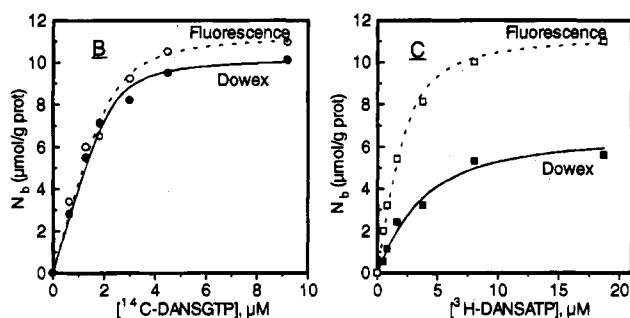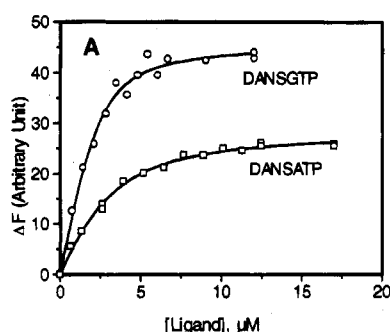
FIGURE 3: Concentration dependence of binding based on the fluorescence titration and the anion exchange method. (A) Titration of 225 μg/mL UCP with DANSGTP and DANSATP; the solid line represents the theoretical fitting with $U_o = 2.5 \pm 0.3$ μM or 11.1 $\pm$ 1.3 μmol/g, $K_D = 0.37 \pm 0.08$ μM for DANSGTP and $U_o = 2.6 \pm 0.5$ μM or 11.7 $\pm$ 2.3 μmol/g, $K_D = 0.83 \pm 0.11$ μM for DANSATP. In another experiment, radiolabeled DANSGTP (B) or DANSATP (C) was added to 200 μg of UCP/mL. After the fluorescence measurement, an aliquot of 50 μL was passed through the Dowex column (see Materials and Methods for binding measurement). The amount of bound DANS nucleotide (μmol/g of protein) from the fluorescence measurements (B and C) was calculated from $N_b = \Delta F/\phi$[mg of UCP/mL]); $B_{max} = 11.2$ μmol/g for DANSGTP and -ATP, $K_D = 0.30$ μM for DANSGTP, and $K_D = 0.85$ μM for DANSATP. From the anion exchange measurements, $B_{max} = 10.8$ μmol/g and $K_D = 0.32$ μM for [^{14}C]DANSGTP; $B_{max} = 6.3$ μmol/g and $K_D = 2.5$ μM for [^{3}H]DANSATP. Fluorescence measurements were made at 360—515 nm in 20 mM Hepes buffer, pH 6.8, at room temperature.

The binding of [^{14}C]DANSGTP and [^{3}H]DANSATP as measured by fluorescence titration, and the anion exchange method was compared as follows. Parallel to the fluorescence titration a portion of the solution was passed through the Dowex column to measure the binding. As shown in

Figure 3B,C, the fluorescence titration with DANSGTP and DANSATP is evaluated on the basis of the binding capacity of [^{14}C]GTP (11.2 μmol/g of protein). The binding capacity of [^{14}C]DANSGTP as measured by the two methods agrees well, and the K_D's are also close; however, the [^{3}H]-DANSATP binding capacity measured by the anion exchange method (Dowex) is only 60% as large as, and the K_D is 3 times larger than, that obtained from the fluorescence measurement. This lower binding capacity of DANSATP as measured by the anion exchange method was observed repeatedly. A plausible explanation for this discrepancy is the existence of loose and tight binding states to the UCP. While the anion exchange method measures only the tight binding portion, fluorescence titration measures the sum of both tight and loose complexes (see Discussion).

Table 1 summarizes the binding parameters for the various DANS and DAN nucleotides measured by fluorescence titration. The dissociation constant of dansylated GTP, ATP, and ADP is smaller than that of the unsubstituted nucleotide measured by equilibrium dialysis (Lin & Klingenberg, 1982) or the anion exchange method (Klingenberg, 1988), whereas DAN-ATP has weaker affinity. Remarkably, although AMP itself binds rather weakly with $K_D = 110$ μM, the affinity increases ~50-fold after substitution by DANS or DAN. The micromolar fluorescence (ϕ) for DANSGTP is nearly 2 times higher than that for DANSATP or DANSADP. When compared with the fluorescence in aqueous solution, the fluorescence enhancement is 30-, 10-, and 10-fold, respectively.

To study the temperature dependence of the dissociation constant, fluorescence titration with DANSATP was performed at temperatures from 2 to 37 °C. The van't Hoff plot (not shown) reveals a rather weak temperature depend-
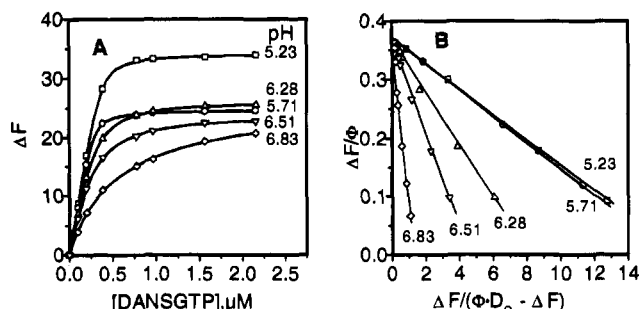
FIGURE 4: pH dependence of fluorescence titration of UCP with DANSGTP. UCP (40 μg/mL) in buffers of pH ranging from 5.2 to 6.8 was titrated with DANSGTP at 10 °C, fluorescence 360—515 nm: (A) titration curves at various pH's and (B) the mass action plots (eq 2, Materials and Methods) where $\Delta F/\phi = N_b$ (μM) and $\Delta F/(\phi D_o - \Delta F) = N_b/N_f$.

ence with $\Delta H = 4.9$ kJ mol^{-1} at temperatures up to 30 °C. Above that temperature, the affinity decreases with temperature, suggesting that UCP might be unstable.

pH Dependence of DANS Nucleotide Binding. The stability of UCP was checked throughout the pH range by control experiments as follows. UCP was incubated in buffers of pH from 1 to 13 for 20 min followed by dilution with a standard buffer and determination of DANSATP binding. The results show that UCP retained its full activity after exposed to pH from 4 to 10 for 20 min at 10 °C.

Typical titrations of UCP with increasing concentrations of DANSGTP at various pH's are shown in Figure 4A. The straight lines in the mass action plots (eq 2) merge at the same maximal binding on the ordinate (Figure 4B). The K_D is estimated from the slope of the mass action plot. For DANSGTP, the K_D increases 11-fold from 0.20 μM at pH 6.8 to 0.018 μM at pH 5.2; in contrast, the fluorescence (ϕ) remains constant at pH 6.8—5.7.

For further evaluation, the pH dependence is given in plots of pK_D against pH. As shown in Figures 5 and 6, solid lines represent theoretical fitting for DANS and DAN nucleotides computed as shown in the following. For comparison, the former theoretical fitting curves (dashed line) for GTP, ATP, and ADP binding are also given (Klingenberg, 1988). At pH < 6.5, DANSNTP has a 10—30-fold higher affinity than NTP, while at higher pH the discrepancy is smaller. Part of the difference is explained by the underestimation of the latter due to competitive inhibition by maleate used in the buffer (Klingenberg, 1988). However, the affinity of DANS nucleotide is distinctly higher at lower pH after correction for the inhibition was made.

For DANSGTP and DANSATP, slopes of -1 at pH $\approx$ 6 and -2 at pH > 7.2 agree with those observed for ATP or GTP (Figure 5). In general, DANSGTP binds somewhat more tightly than DANSATP. This is indicative of an additional interaction of the C-6 oxygen on the guanine moiety; participation of this oxygen in hydrogen bonding to EF-Tu was reported (Schimmel, 1993). DANSADP binds 3—5 times more tightly than ADP, and both exhibit the same slopes, i.e., -1 at pH > 7 and 0 at pH < 6. The affinity of AMP after dansylation increases 50 times (pH 6.5). The K_D can be determined from fluorescence titrations, whereas AMP itself binds too weakly to be evaluated directly by equilibrium dialysis or the anion exchange method. The pK_D for DANSAMP is fairly constant between pH 6 and 7.5 but decreases at higher pH with a slope $\approx$ -1. Also at lower
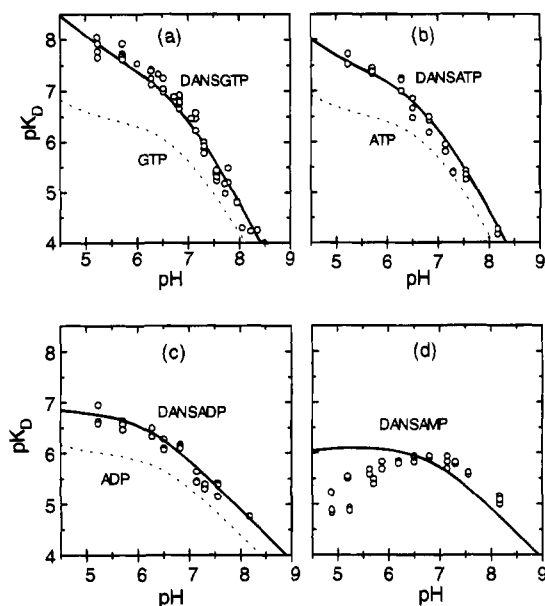

FIGURE 5: pH dependence of dissociation constants of DANS nucleotides binding to UCP. The K_D values were obtained from fluorescence titrations for the DANS nucleotides (conditions as for Figure 4). The theoretical pK_D/pH curves (solid line) were drawn from equations with the literature K_H values (Appendix) and intrinsic dissociation constants (Table 2). The pK_D/pH curves (dashed line) for the unsubstituted nucleotides were taken from Klingenberg (1988).
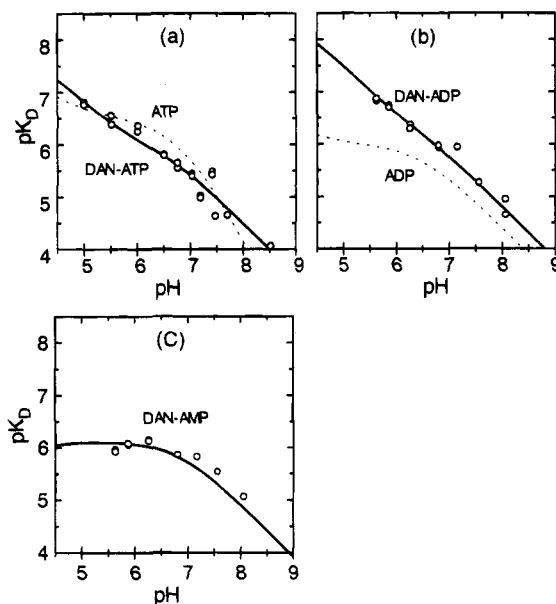

FIGURE 6: pH dependence of dissociation constants of DAN nucleotides binding to UCP. The K_D values were obtained from fluorescence titrations for the DAN nucleotides. The theoretical pK_D/pH curves (solid line) were drawn from equations with the literature K_H values (Appendix) and intrinsic dissociation constants (Table 2).

pH, the pK_D tends to decrease. In contrast, the pK_D/pH plots for both DAN-ATP and DAN-ADP show straight lines of slope -1 within the pH range investigated, and they have similar affinities (Figure 6). The pH dependence of the affinity of DAN-AMP is similar to that of DANSAMP.

Previously a model was developed for explaining the pK_D/ pH curves of the unsubstituted nucleotides (Klingenberg, 1988). Three H$^+$ dissociations were introduced, one at the terminal phosphate of the nucleotide and two at the protein

Table 2: Intrinsic Dissociation Constants for the Fluorescent Nucleotide Derivatives[a]

	intrinsic dissociation constants (M)	
	K_{NH}	K_N
DANSATP	2×10^{-9}	2×10^{-10}
DANSGTP	1.2×10^{-8}	1.2×10^{-10}
DANSADP	8×10^{-8}	8×10^{-10}
DANSAMP	∞	7.5×10^{-10}
DAN-ATP	1×10^{-8}	2×10^{-9}
DAN-ADP	2×10^{-9}	1×10^{-9}
DAN-AMP	∞	7.5×10^{-10}

[a] The constants were obtained from the best fitting of the pK_D versus pH plots (Figures 5 and 6). K_{NH} and K_N designate the intrinsic dissociation constants of DANSNTPH^{3-} and DANSNTP^{4-}, etc. (see also text).
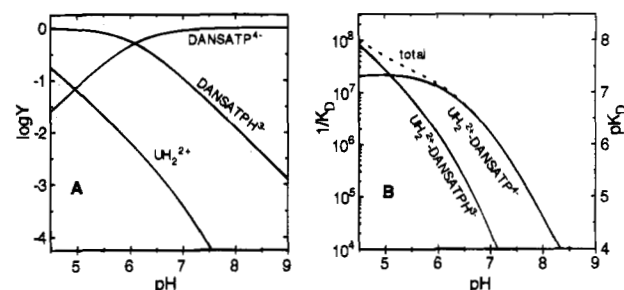

FIGURE 7: pH dependence of the relative concentration of the nucleotide binding species and their contributions to the total binding affinity. (A) The concentration dependence of the two species for DANSATP was computed using $pK_{H3} = 6.5$ for protonation at the γ-phosphate and for UH$_2^{2+}$ $pK_{H1} = 3.8$ for the carboxylate group of Glu/Asp and $pK_{H2} = 7.2$ for the His (Klingenberg, 1988). (B) Contribution of the intrinsic affinity to the total affinity: K_{NH} represents the dissociation constant of DANSATPH^{3-}, K_N of DANSATP^{4-}. The individual intrinsic dissociation constants of these species were obtained from fitting the pK_D/pH plots.

binding site. With this model, good fit of the pK_D/pH curves was obtained whereby the intrinsic binding constants were estimated for the unsubstituted nucleotides. Using the same procedure, we obtain good fit of the pK_D/pH curves for the DANS-substituted nucleotides (solid lines in Figure 5). The intrinsic dissociation constants are summarized in Table 2.

As shown in Table 2, the intrinsic dissociation constants can be compared to those of the unsubstituted nucleotides, e.g., $K_{NH} = 2 \times 10^{-9}$ (DANSATPH^{3-}) versus 4.5×10^{-8} (ATPH^{3-}), $K_N = 2 \times 10^{-10}$ (DANSATP^{4-}) versus 9×10^{-10} (ATP^{4-}) and $K_{NH} = 8 \times 10^{-8}$ (DANSADPH^{2-}) versus 2.5×10^{-7} (ADPH^{2-}), $K_N = 8 \times 10^{-10}$ (DANSADP^{3-}) versus 2.5×10^{-9} (ADP^{3-}). As explained above, the general higher affinity of the DANS nucleotides is partially due to the absence of competing anions such as maleate. DANSNTP^{4-} binds about 10 times more strongly than DANSNTPH^{3-} and DANSADP^{3-} 100 times more strongly than DANSADPH^{2-}. Klingenberg (1988) reported 50 and 100 times higher affinity of NTP^{4-} over NTPH^{3-} and ADP^{3-} over ADPH^{2-}, respectively. It appears likely that dansylation might have enhanced the affinity of the protonated species. For DANSAMP as well as DAN-AMP, we assume that only DANSAMP^{2-} is the sole binding species. With this assumption the calculated curve fits well to the points at pH > 6.5 but poorly to those at lower pH as it tends to be flat at lower pH. The weaker affinity at low pH is still unknown, due to some further factors. The peculiar pK_D/pH relationships for DAN-ATP and DAN-ADP have been best fitted assuming that the His is not involved in the binding and that the affinity of DAN-ATPH^{3-} and DAN-ATP^{4-} is close (Table 2).

To better illustrate the pH dependence of binding, the distribution of the binding species in dependence on pH for DANSATP is shown in Figure 7A. DANSATP binds the UCP in two forms, i.e., DANSATPH^{3-} and DANSATP^{4-}. The intrinsic dissociation constants are denoted K_{NH} and K_N, respectively. Only the form UH$_2^{2+}$ where both the Glu/Asp and His residues are protonated is plotted, since it must be assumed that only this form can bind the triphosphate. The variation of the distribution of each species in dependence on pH is shown in logarithmic terms (log Y). It is thus obvious that as pH increases the concentration of UH$_2^{2+}$ decreases with slope $= -1$ at pH 4–6.5 due to the deprotonation at the carboxylate of Glu/Asp and with slope $= -2$ at pH > 7.2 due to the further deprotonation of the HisH$^+$ residue. In other words, more and more UCP will be in the nonbinding form, but DANSATP is increasingly

deprotonated into the higher affinity species DANSATP^{4-}. The logarithmic sum is mainly dominated by the UH$_2^{2+}$ term, and thus a slope of -2 is observed at higher pH. Figure 7B illustrates the contributions of the two species to the total binding affinity. The apparent total dissociation constant can be decomposed as follows: $1/K_D = 1/K_{NH} + 1/K_N$. The solid lines in Figure 7B represent the affinity ($1/K_{NH}$ or $1/K_N$) of the individual species in dependence on pH, and the algebraic sum of these contributions gives the apparent total affinity, $1/K_D$ (dashed line), of DANSATP. These lines have been computed with the intrinsic dissociation constants obtained from the fitting (Table 2). The contribution by DANSATPH^{3-} is mainly at lower pH. DANSATP^{4-} shows 10 times higher affinity for UH$_2^{2+}$. At higher pH, DANSATP^{4-} contributes solely to the total affinity, which is only limited by the inactivation of UCP. As a result, the total K_D (dashed line) is relatively constant at pH < 6 and falls with increasing steepness up to $\Delta pK_D/\Delta pH = -2$ at high pH.

Influence of Ions. As expected there is a strong decrease in the affinity of DANSATP for UCP in the presence of Mg^{2+}. Fluorescence titrations yield an inhibition constant of 21.4 ± 0.4 μM, which corresponds to a stability constant of 4×10^4 M^{-1}. This value is of the same order of magnitude as compared with the literature data for the Mg-ATP complex (Bock, 1960).

The affinity of DANS nucleotide is lowered in the presence of anions, in a similar manner as that reported for the unsubstituted nucleotide (Klingenberg, 1988). The results are summarized in Table 3. As will be shown in the kinetic studies, anions decrease the affinity by binding to the UCP and hence decrease the nucleotide binding sites.

Probing the Affinity of Nucleotide and Analogues. Since most nucleotide analogues are not available in the radiolabeled form, DANS nucleotides provide convenient probes for measuring the affinities of nucleotides and their analogues by competition. First, UCP was incubated with the nucleotide, and then the fluorescence on titration with increasing amounts of DANSATP was measured. In all the cases studied, competitive inhibition of DANS nucleotide binding was observed. Therefore the measured inhibition constant (K_i) should correspond to the dissociation constant (K_D). The results are summarized in Table 4. For comparison, some

Table 3: Inhibition by Ions of DANSATP Binding to UCP[a]

ion	K_i (mM)
Mg^{2+}	0.021
pyrophosphate	0.16
sulfate	1.0
phosphate	4.4
maleate	8.0
chloride	9.3
acetate	large

[a] Fluorescence titrations of 40 μg/mL UCP were carried out at pH 6.8 and 10 °C in the presence of the ion. In all the cases, competitive inhibition of DANSATP binding by the ion was observed. The inhibition constants were obtained from the double-reciprocal plots.

Table 4: Specificity of Nucleotide Binding Affinity as Measured by Competition with DANSATP[a]

inhibitor	K_i (μM)	inhibitor	K_i (μM)
ATP	0.20 (3.1)	APS	49
d-ATP	0.17	PAP	2.0
AMPPNP	3.0	AMP	110 (424)
AMPPCP	11.5	3'-AMP	480
ATP-γS	1.14	GTP	0.24 (1.4)
ATP-P	12	GDP	0.50 (2.5)
AP$_5$A	7.4	GMP	30 (110)
ADP	1.52 (4.9)	3'-GMP	90
d-ADP	1.52	UDP	7.6
8-Br-ADP	1.5	palmitoyl CoA	13

[a] Fluorescence titrations of 40 μg/mL UCP were carried out at pH 6.8 and 10 °C in the presence of the nucleotide or analogue. In all the cases, competitive inhibition of DANSATP binding was observed. The inhibition constants were obtained from the double-reciprocal plots. For comparison, data obtained by equilibrium dialysis (Lin & Klingenberg, 1982) are included in the parentheses.

former data (Lin & Klingenberg, 1982) are also listed which were obtained by equilibrium dialysis. The K_D values are 3—10-fold larger than those measured in this work. A plausible reason for this discrepancy is that the reported K_D was determined in buffers containing 20 mM Na_2SO_4, which inhibits nucleotide binding. For example, using $K_i = 1.0$ mM, the dissociation constant for ATP would be 0.15 μM when calculated from 3.1 μM. This agrees well with the value (0.20 μM) from the fluorescence measurement. Also in the buffer containing 25 mM maleate (Klingenberg, 1988), the affinity of nucleotides is lowered due to competition by maleate ($K_i = 8.0$ mM, Table 3).

As seen in Table 4, an elongated phosphate portion such as in ATP-P and palmitoyl CoA does not fit well to the binding site since they show 60—70-fold lower affinity. The 2'-hydroxyl group does not contribute to the binding since the deoxy nucleotides are equally bound and DANS substitution at this position does not change the binding properties. The ATP analogues, AMPPNP, AMPPCP, and ATP-γS, have reduced affinities in comparison to ATP, due to altered pK_H of the γ-phosphate as discussed in Klingenberg (1988). The ADP analogues, 8-bromo-ADP, PAP, and ADP-ribose, bind similar to ADP, while substitution of the β-phosphate with sulfate (APS) results in a 40-fold decrease in affinity. The affinity of 5'-AMP and 5'-GMP is nearly 3 times higher than that of 3'-AMP and 3'-GMP. The other nucleotides, UDP and TDP, and their analogues show low affinities, in accordance with the early observation (Lin & Klingenberg, 1982).

Chemical Modification. The influence of some amino acid reagents on nucleotide binding of UCP is monitored by

Table 5: Summary of the Effects of Chemical Modification of UCP as Probed by DANSGTP[a]

target residue	reagent	pH	conc (mM)	required incubation time (min)	residual UCP (%)
Asp/Glu	Woodward's reagent K	6.8	0.030	15	9.4
Arg	phenylglyoxal	8.0	0.9—2.4	10	22—24
	cyclohexanedione	8.4	0.25—3.0	130	79—36
Lys	TNBS	8.0	1.0	10	50
His	diethylpyrocarbonate	8.0	3.0	20	70
Cys	mersalyl	8.0	0.012—1.5	5	88—13
	HMB	8.0	0.005—0.09	5	94—9
	pCMS	7.2	0.025—0.05	10	70—10

[a] Chemical modification of the UCP was carried out at room temperature. First, UCP was incubated with the reagent at the indicated pH and reagent concentration. The given incubation time ensured complete reaction as demonstrated by the time dependence measurements under the conditions. Then, the UCP was diluted at least 10-fold into a reference buffer (20 mM Hepes, pH 6.8), and fluorescence titration with DANSGTP was made (see the text). TNBS, trinitrobenzenesulfonic acid; HMB, hydroxymercuribenzoic acid; pCMS, p-chloromercuribenzenesulfonic acid.

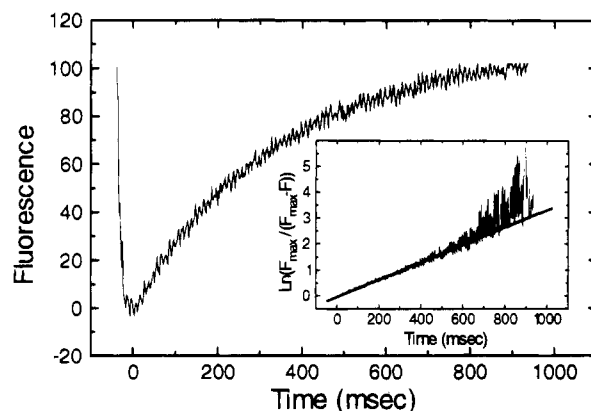FIGURE 8: Fluorescence transient for the binding of DANSATP to UCP as recorded on the stopped flow mixing apparatus. DANSATP (5 μM) was added to 0.5 μM UCP in 20 mM Mes buffer, pH 6.3, at 23 °C. Inset: plot according to the first-order rate equation $\ln[F_{max}/(F_{max} - F)] = kt$.

titration with DANSGTP. The results are summarized in Table 5. Modification of residues of Glu/Asp, His, Arg/Lys, and Cys decreases nucleotide binding. In all the cases, fluorescence titration of the reaction mixture did not influence the K_D but only the ΔF_{max}. The ΔF_{max} should correspond to the residual UCP. The Woodward reagent eliminated binding of all the nucleotide derivatives including DANSAMP and DAN-AMP to the same extent. Diethylpyrocarbonate inactivated DANSGTP binding only weakly. At a reagent concentration of 3 mM, still 71% UCP was active. Although mercurials impaired nucleotide binding, modification with N-ethylmaleimide did not inactivate. This indicates that the essential Cys may be in a hydrophilic environment. The reagents for tyrosine and serine did not inhibit nucleotide binding.

Kinetics of Interaction of DANS Nucleotide with UCP. The fluorescent nucleotide derivatives offer the opportunity for transient kinetic studies. A typical recording of the kinetics of binding is shown in Figure 8. When the fluorescence signal was linearized according to the first-order rate equation $\ln[F_{max}/(F_{max} - F)] = kt$, the data fitted well to a straight line which bent upward only at time >700 ms (inset of Figure 8). The pseudo-first-order rate constant was evaluated from the slope. To determine the concentra-

Table 6: Rates of Binding and Dissociation of DANS and DAN
Nucleotides at pH 6.3 and 23 °C[a]

ligand	$k_{on} \times 10^{-6}$ $(M^{-1} s^{-1})$	k_{off} (s^{-1})	$K_D = k_{off}/k_{on}$	K_D (μM)
DANSGTP	0.36	0.026	0.073	0.042
DANSATP	0.40	0.044	0.11	0.091
DANSADP	0.31	0.41	1.32	0.65
DAN-ATP	0.30	0.41	1.37	1.04
DAN-ADP	0.30	0.40	1.33	0.87

[a] The rate constants were determined on the stopped flow fluorescence photometer. K_D values obtained from fluorescence titrations are given for comparison.
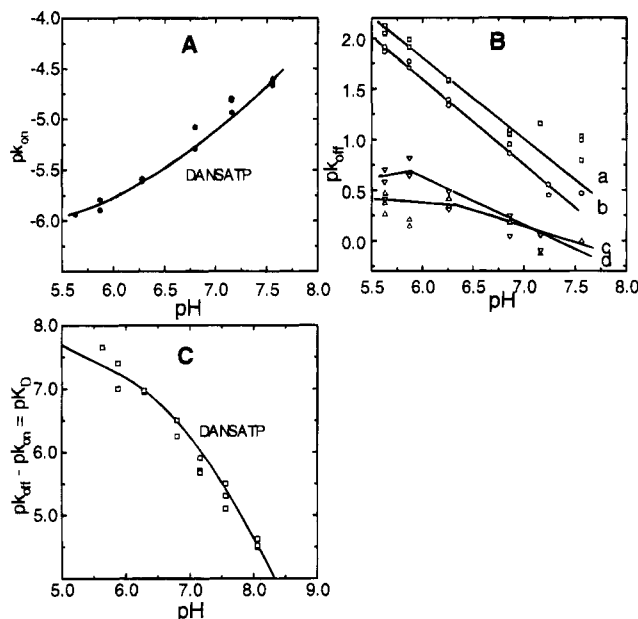

FIGURE 9: pH dependence of the binding and dissociation rates. pH dependence of pk_{on} (A) for DANSATP and of pk_{off} (B) for (a) DANSGTP, (b) DANSATP, (c) DANSADP, and (d) DAN-ATP. (C) pK_D/pH plot for DANSATP where the K_D was calculated from the kinetic measurements $(K_D = k_{off}/k_{on})$; the theoretical curve (see also Figure 5) is also plotted. Measurements in 20 mM Mes or Hepes buffer at 23 °C.

tion dependence of the binding rate, the concentration ofDANSATP was varied. A plot of the pseudo-first-order rate constant against DANSATP concentration (not shown) gives a straight line with second-order rate constant $k_{on} = 4.0 \times 10^5 M^{-1} s^{-1}$. As shown in Table 6, the binding rate constants for all fluorescent nucleotide derivatives are quite similar. The dissociation is followed by the fluorescence decrease on addition of large excess ATP, and analysis of the recordings suggests that it obeys a first-order reaction. In contrast to the binding rate, the dissociation rates differ widely depending on the nucleotide, e.g., DANSGTP dissociates most slowly, DANSATP slightly faster, but the others about 16 times faster.

The pH dependence of the binding and dissociation rate constant is plotted in Figure 9. As measured for DANSATP, the binding rate decreases with pH, showing a slope ≈ 1 in the pk_{on}/pH plot. At lower pH, the slope bends presumably because of decreasing $DANSATP^{4-}$ concentration (Figure 9A). To the contrary, the dissociation rate of DANSNTP increases as the pH increases with a $\Delta pk_{off}/\Delta pH \approx -1$ (Figure 9B). The K_D values for DANSATP as calculated from the ratio k_{off}/k_{on} fit well to the theoretical pK_D/pH curve (Figure 9C). DANSGTP dissociates more slowly than
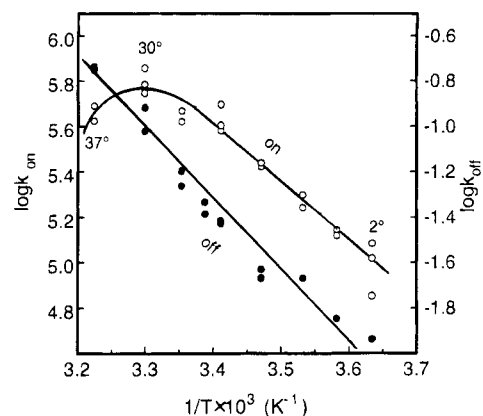

FIGURE 10: Arrhenius plot for the temperature dependence of the binding and dissociation rates of DANSATP. The on rate was measured with the stopped flow fluorometer and the off rate determined with the fluorescence spectrophotometer in 20 mM Mes buffer at pH 6.3.

DANSATP in the whole pH range studied. However, the dissociation of DANSADP and DAN-ATP is rapid and exhibits low pH dependency.

Generally anions decrease the rate of DANS nucleotide binding but not that of dissociation. The binding rate of DANSATP decreased 20-fold in 20 mM sulfate and 4-fold in 25 mM maleate; interestingly, we observed little influence on the binding rate in the presence of 40 mM acetate. The results are consistent with the decrease in the binding affinity as demonstrated above by competition studies. This reveals that the anions decrease the binding rate or affinity by occupying the binding sites of the UCP.

The temperature dependence of log k_{on} shows a biphasic shape. At temperatures above 30 °C, the binding rate tends to decrease, indicating that UCP was partially denatured. Below 30 °C, it increases with temperature with an energy of activation of 54 kJ mol^{-1} (Figure 10). The dissociation rate increases with temperature showing an energy of activation of 69 kJ mol^{-1}.

DISCUSSION

(Dimethylamino)naphthalene-substituted nucleotides have been shown to be very useful fluorescent probes for nucleotide binding to two related mitochondrial carrier proteins, the ADP/ATP carrier and the uncoupling protein. The (dimethylamino)naphthoic acid nucleotide derivatives were used to monitor the functional state of the carrier sites in the AAC (Mayer et al., 1984; Klingenberg et al., 1984, 1985) and, also to some extent, the nucleotide binding site in the UCP (Klingenberg, 1984). The fluorescence of DAN-ATP is strongly quenched in aqueous solutions and increases dramatically on binding. Unsubstituted naphthoic acid derivatives of ADP and ATP were also applied for studying the AAC (Block et al., 1979, 1982; Mayer et al., 1984); however, they proved to be much less useful since the fluorescence of the naphthoic acid derivatives of ADP and ATP is decreased on binding. The dansyl derivatives share the advantages of strong fluorescence increase with the DAN derivatives. However, they do not bind to the AAC but interact more strongly with the UCP. For this reason the use of the DANS-substituted nucleotides is restricted primarily to the UCP.

Binding of DANS Nucleotides to UCP. The competition studies clearly show that the DANS nucleotide derivatives bind to the same site as the unsubstituted nucleotides. Also, the binding affinity of the DANS nucleotides has a strong pH dependence similar to that of the nucleotides. Whereas the affinity of the DAN nucleotides is markedly lower, the affinity of DANSATP and DANSGTP even surpasses that of ATP and GTP. For example, at pH 6.5 the K_D's are 0.040 μM for DANSGTP and 0.20 μM for GTP. The strong increase of affinity (50-fold) on substituting AMP with a DANS or DAN group illustrates the contribution of hydrophobic interaction of DANS and DAN groups to the binding. Interestingly a similar observation was reported for DAN nucleotide binding to the AAC; for the virtually nonbinding AMP the enhancement was even 100-fold (Klingenberg et al., 1985). For ATP, GTP, or ADP, the binding affinity increase by the substitution is obvious at lower pH. The results of fluorescence titrations show that DANSGTP and DANSATP bind 11.4 μmol/g of protein. Although this value is lower than the maximum expected [16 μmol of GDP/g of protein, Lin and Klingenberg (1980)] due to partial impurity, it agrees with the corresponding [^{14}C]GTP binding. We conclude that the DANS nucleotides bind with a stoichiometry of 1 mol to 1 mol of dimeric UCP.

The fluorescence measurements are verified with radiolabeled DANS nucleotides using the anion exchange method. Whereas for DANSGTP the binding measured with fluorescence titrations agrees with the anion exchange method, [^{3}H]DANSATP binding is ~40% lower than that determined by the fluorescence titration. A plausible explanation for this discrepancy is the existence of a mixed population of tightly and loosely bound UCP—nucleotide complexes which are in a slowly exchanging equilibrium. The fluorescence titration measures the total sum of the tight and loose complexes, while the rapid anion exchange allows segregating only the tight complex. The ratio of the tight and loose complexes differs widely between the derivatives. While binding of DANSGTP, GTP, and ATP forms largely the tight complex, DANSATP binding results in formation of only 60% tight complex. This aspect will be elucidated in context with the varying ability of the nucleotide derivatives to inhibit H$^+$ transport and induce conformational changes in the protein, to be reported in a subsequent paper.

The difference in binding affinities between the DANS and DAN nucleotides could be due to the different substitution sites on the ribose as shown by the NMR spectra. Whereas DANSATP is exclusively present as the 2'-O-substituted form, DAN-ATP exists as 60% 3'-O- and 40% 2'-O-substituted forms. According to energy minimization calculations, in the 2'-O-substitution the phosphate is inclined toward the dansyl group and in the 3'-O-substitution it is straight (Figure 11). We assume these differences are responsible for the high affinity of the DANS nucleotides because they fit better into the binding pocket.

pH Dependence of Binding. Most revealing and important for understanding the nucleotide binding to UCP has been the characteristic pH dependence (Klingenberg, 1988). In the present studies with DANS nucleotides, basically a similar pH dependence is observed. In general, the K_D increases with the pH. Characteristic changes in the slopes of the pK_D/pH plots allowed differentiation of the participation of different protonating groups in the nucleotide—UCP interaction (Klingenberg, 1988). Besides the protonation of
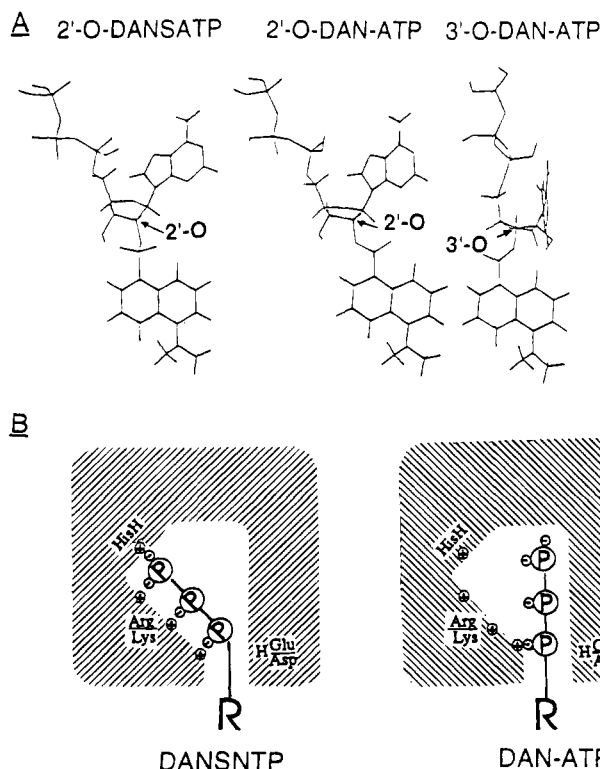

FIGURE 11: (A) Structures of 2'-*O*-DANSATP and 2'-*O*- and 3'-*O*-DAN-ATP. The most relaxed structures have been computed with the DTMM program (desktop molecular modeller, Oxford University Press). The energy minimization works as follows: the program provides an equation describing the energy of the molecule as a function of its coordinates. Then the conformation is adjusted to lower the energy value of the system. A minimum can be found after iterations of the process. The force fields used consist of bond-stretching potential, bond angle deformation potential, periodic torsional barrier potential, and nonbonding interactions for non-bonded atom pairs (van der Waals potential, Coulombic potential, and hydrogen bonding). Molecular dynamics is included to avoid trapping the nucleotide conformations in local minima. The calculations do not include the interactions with the solvent and should therefore approximate the most relaxed structures in vacuum. (B) Model for DANSNTP and 3'-*O*-DAN-ATP bound to UCP. The UCP center is drawn to better illustrate the proposed difference for the DANS and DAN nucleotides. The proposed nucleotide binding center is only speculative but emphasizes the role of the Glu/Asp, His, and Arg/Lys residues in nucleotide binding (see also Figure 12).

the terminal phosphate group of the nucleotide, protonation at the UCP of a carboxylate group and also of a histidine group were postulated by which the pK_D/pH plots could be well fitted. This led to a model of the binding site surrounding the diphosphate or triphosphate moieties of the nucleotides in which the protonation of the CO_2^- group abolishes a charge pair which controls the access to the binding pocket of the phosphate moiety.

The pK_D/pH plots for the DANS nucleotides have been fitted using the same model. The intrinsic dissociation constants are basically comparable with those of the unsubstituted nucleotides. We note a lower affinity of DANSNTP than DANSADP at pH > 7.2, similar as for ATP and ADP (Klingenberg, 1988). This indicates that when the HisH$^+$ is deprotonated, DANSNTP may not be accepted. It can be visualized that on protonation the HisH$^+$ is repelled by a nearby positive charge, thus enlarging the binding cleft to accept the additional phosphate in NTP or DANSNTP (Figure 12). The energy cost of this local rearrangement is
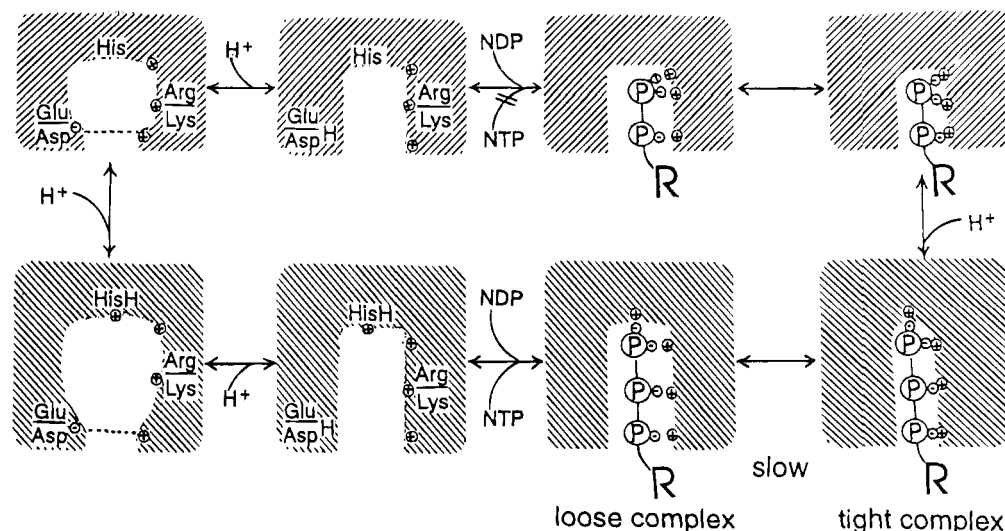
FIGURE 12: Proposed scheme of binding/dissociation reaction at the nucleotide binding site of UCP based on the pH dependence of K_D, k_{on}, and k_{off}. The model [based on Klingenberg (1988)] postulates that the interaction of the nucleotide phosphate moiety with positive residues of UCP is controlled by a carboxylate group which forms via ion pair a gate to the binding niche. On protonation of the carboxylate group, the gate opens and the positive residues become accessible. This results in a slope $\Delta pK_D/\Delta pH = -1$ dependency. To this relation is overimposed the protonation with a $pK_H \approx 6.4$ of the terminal nucleotide phosphate with the stipulation that the higher charged form has a higher pK_D ($NTP^4 > NTPH^{3-}$, $NDP^{3-} > NDPH^{2-}$). As a result of these two counteracting influences, the pH dependence of pK_D is partially neutralized at pH $<$ pK_H. The increasing steepness of pK_D at pH $>$ pK_H observed only with NTP is explained by invoking a third proton-accepting residue (e.g., His) with a $pK_H = 7.2$ necessary for binding NTP^{4-} only. The deprotonation at pH $>$ 7.2 of both the Asp/Glu and the His adds up to a steep $\Delta pK_D/\Delta pH = -2$ for binding of NTP only. For NDP^{3-}, the His is not involved in accordance with the slope of -1 at pH $>$ 7.2. The binding niche can be enlarged on protonation of the His in order to accommodate the nucleoside triphosphate. Two types of binding complexes are depicted in accordance with the binding measurements, a loose nucleotide–UCP complex (shown with a broader cleft) and a tight complex (narrower cleft).

compensated by the additional Coulombic binding energy of the triphosphate. Thus the involvement of His produces more a regulatory advantage than additional binding energy. Deprotonation at this $HisH^+$ increases the pH dependence of the nucleoside triphosphate such that the affinity decreases with $[H^+]^{-2}$ above pH 7. On the other side, at low pH, curve fitting requires that the binding also of $DANSNTPH^{3-}/DANSADPH^{2-}$ contributes—although with lower affinity—to the total binding [see also Klingenberg (1988)]. The $pK_D/$pH plot for DANSAMP/DAN-AMP (Figures 5 and 6) has a flat maximum between pH 6 and 7.5. We assume that predominantly $DANSAMP^{2-}/DAN-AMP^{2-}$ but not $DANSAMPH^-/DAN-AMPH^-$ binds to UCP. Thus the relative affinity of the species with protonated terminal phosphate is reduced increasingly going from NTP ($K_{NH}/K_N = 10$) to ADP ($K_{NH}/K_N = 100$) to AMP ($K_{NH}/K_N = \infty$).

The $pK_D/$pH relationship for the DAN nucleotides is confined to a nearly 1 order lower affinity level than for the DANS nucleotides at pH $<$ 7.0. The absence of a break is explained by assuming an about equal binding affinity of the two dissociation forms $DAN-ATPH^{3-}$ and $DAN-ATP^{4-}$ and that the $HisH^+$ is not involved in the binding. The reason may be the conformational difference, where the 3'-O-DAN substitution results in a wider gap between the γ-phosphate and the $HisH^+$ (Figure 11B).

Kinetics of Binding. The use of fluorescent nucleotide derivatives permits kinetic studies. The binding rates for the four fluorescent nucleotides are nearly equal (4×10^5 $M^{-1} s^{-1}$). In contrast, the dissociation rate is more characteristic of the nucleotide derivatives. To interpret the binding and dissociation rates and their pH dependency, a model is presented in Figure 12, which is a further development of the earlier version based on the equilibrium binding measurements (Klingenberg, 1988). Two additional features are

incorporated (1) a binding niche enlarged on protonation of the His for nucleoside triphosphate binding and (2) the existence of a loose and tight nucleotide–UCP complex, the transition between both being slow. As before, nucleotide binding is allowed only if the carboxylate group of the Glu/Asp is protonated.

The binding reactions are formulated as follows,
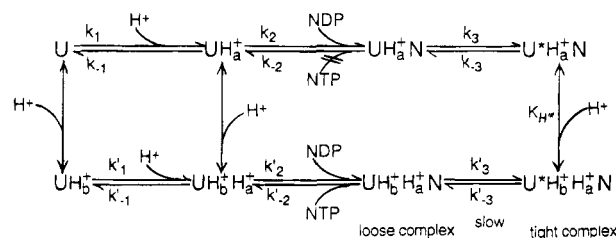
where the two H^+ binding sites are designated H_a^+ for Glu/Asp and H_b^+ for His. According to this scheme, the binding rate measures the binding first to the loosely binding complexes (UH_a^+N or $UH_b^+H_a^+N$). Limiting is the low concentration of UH_a^+ since from the binding studies the protonation was calculated to be at $pK_H \approx 3.8$ (Klingenberg, 1988). Considering the upper pathway in the scheme, one can write the binding rate as

$$V_{on} = k_2[UH_a^+][N] = k_2[U_o][N][H^+]/(K_{Ha} + [H^+])$$

The measured second-order rate constant k_{on} is in dependence on the H^+ concentration,

$$k_{on} = V_{on}/[U_o][N] = k_2[H^+]/(K_{Ha} + [H^+])$$

where $[U_o]$ is the total UCP concentration; K_{Ha} describes the deprotonation at the Glu/Asp. In the pk_{on} versus pH plot,

this should give a slope of 1 since $K_{Ha} \gg$ [H$^+$], which is in agreement with the results for DANSATP (Figure 9A). The protonation of UH$_b^+$ is not critical since it occurs at a pK_H between 7 and 8. The flat pH dependence below pH 6 is explained by the decreased portion of DANSATP^{4-}.

The dissociation rate k_{off} is measured by the trapping of the dissociated complex with excess ATP. The dissociation rate can reflect either the slow transition from the tight to the loose binding state or the dissociation from the loose binding state, depending on the nucleotide involved. The dissociation rate for the tight binding DANSNTP is about 16 times slower than those for the more loosely binding DANSADP and DAN-ATP. In fact, the ratio of the k_{on} and k_{off} for the various nucleotides agrees quite well with the K_D measured by fluorescence titration. This is an important verification of the K_D determined by equilibrium measurements and vice versa of the dissociation and binding rates.

In terms of the reaction scheme, we may conclude that the k_{off} in the case of DANSNTP is determined primarily by k_{-3} and for the more loosely binding species DANSADP and DAN-ATP by k_{-2}. The k_{off} of the more tightly binding DANSNTP has a strong pH dependence with Δpk_{off}/ΔpH ≈ -1, whereas the k_{off} of the loosely binding nucleotides has only a fractional pH dependence.

Alternative routes for the dissociation can be visualized according to this scheme. First the tight nucleotide complex U*H$_b^+$H$_a^+$N would slowly return to the loose complex UH$_b^+$H$_a^+$N which readily dissociates. However, in this case no pH dependence can be expected since the H$^+$ dissociation occurs after the rate-limiting slow conformation transition. A second route involves first a deprotonation from the tight complex and then the formation of the loose complex: U*H$_b^+$H$_a^+$N $\rightarrow$ U*H$_a^+$N $\rightarrow$ UH$_a^+$N. In this case the dissociation rate is limited by the amount of the tight complex U*H$_a^+$N which can be expected to be very low. Thus the pH dependency of the dissociation rate can be expressed as

$$V_{off} = k_{-3}[\text{U*H}_a^+\text{N}] = k_{-3}K_{Hb}[\text{U*H}_b^+\text{H}_a^+\text{N}]/[\text{H}^+]$$

and rearranged to conform with the pk_{off} versus pH plot,

$$k_{off} = V_{off}/[\text{U*H}_b^+\text{H}_a^+\text{N}] = k_{-3}K_{Hb}/[\text{H}^+]$$

where $K_{Hb} = [\text{U*H}_N^+][\text{H}^+]/[\text{U*H}_b^+\text{H}_a^+\text{N}]$ describes the H$^+$ dissociation of the tight complex with a K_H which can be well below 10^{-9} M. As a result, the pk_{off}/pH should give a line with a slope of -1 in the observed range.

Which of the two deprotonation sites limits the dissociation rate can be concluded from the fact that the strong pH dependence is observed only for the dissociation of DANSNTP but not for DANSADP. This suggests that the deprotonation at the HisH$^+$, i.e., the level of [U*H$_a^+$N], limits the V_{off}. One might argue that this is improbable since the pK_H of the His is much higher than that of the Asp/Glu group. However, in the bound complex the pK_H of the Asp/Glu group might be strongly down shifted.

Thermodynamics of Binding. From the temperature dependence of the rate of DANSATP binding, an activation energy of 54 kJ mol^{-1} is determined. The activation energy for dissociation is higher (69 kJ mol^{-1}). The free energy change (ΔG) for DANSATP is -33.6 kJ mol^{-1} as calculated from the dissociation constant K_D. The van't Hoff plot

reveals a small positive enthalpy change which amounts to 4.9 kJ mol^{-1}. From these, an entropy change (ΔS) of 136 J deg^{-1} mol^{-1} is calculated. These data suggest that enthalpy change contributes little to the binding, which is driven primarily by the entropy change. The rather large ΔS value may be interpreted as due to strong hydrophobic and ionic (charge neutralization) interactions associated with removal of water molecules from the binding site (Ginsburg et al., 1987; Ross & Subramanian, 1981).

The thermodynamic data resemble quantitatively those for AMPPNP or ATP binding to glutamine synthetase (Ginsburg et al., 1987) and AMPPNP binding to myosin (Swenson & Ritchie, 1979). This may be indicative of a common feature of nucleotide binding to proteins. The activation energy (54 kJ mol^{-1}) for DANSATP binding to UCP can be compared to the activation energy of $\sim$50 kJ mol^{-1} for ATP binding to myosin (Bagshaw & Trentham, 1974; Ouellet et al., 1952).
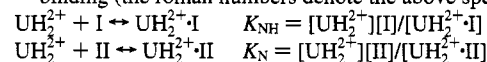
ACKNOWLEDGMENT

APPENDIX

The purpose is to derive the relation of K_D to H$^+$ concentration. Consider the binding of DANSNTP (N = G or A) to UCP. In the following, U and D stand for [UCP] and [DANSATP^{4-}]. Thus the dissociations and binding involved are written in the following with the corresponding constants,

dissociations	site	pK_H	literature
UH$_2^{2+}$ $\leftrightarrow$ UH$^+$ + H$^+$	Asp/Glu	pK_{H1} = 3.8	Klingenberg (1988)
UH$^+$ $\leftrightarrow$ U + H$^+$	His	pK_{H2} = 7.2	Klingenberg (1988)
DANSATPH^{3-} $\leftrightarrow$	γ-Pi	pK_{H3} = 6.5	Klingenberg (1988)
(I)			
DANSATP^{4-} + H$^+$			
(II)			

binding (the roman numbers denote the above species of ligand)
UH$_2^{2+}$ + I $\leftrightarrow$ UH$_2^{2+}$·I K_{NH} = [UH$_2^{2+}$][I]/[UH$_2^{2+}$·I]
UH$_2^{2+}$ + II $\leftrightarrow$ UH$_2^{2+}$·II K_N = [UH$_2^{2+}$][II]/[UH$_2^{2+}$·II]

The sum of the modes of intrinsic binding is thus as follows (X = [H$^+$]),

$$B = [\text{UH}_2^{2+}\text{·I}] + [\text{UH}_2^{2+}\text{·II}] =$$
$$UD(X^3/K_{H1}K_{H2}K_{H3}K_{NH} + X^2/K_{H1}K_{H2}K_N)$$

The concentrations of free UCP and ligand are

$$U_o - B = \text{U} + \text{UH}^+ + \text{UH}_2^{2+} =$$
$$U(1 + X/K_{H2} + X^2/K_{H1}K_{H2})$$

$$D_o - B = [\text{I}] + [\text{II}] = D(X/K_{H3} + 1)$$

Thus according to the definition, $K_D = (U_o - B)(D_o - B)/B$, and rearranging, we have

$$K_D = \frac{(K_{H1}K_{H2} + K_{H1}X + X^2)(X + K_{H3})}{X^3/K_{NH} + K_{H3}X^2/K_N}$$

Derivation of the binding equations for the other nucleotide derivatives is similar and hence omitted. The results are written for reference.

DANSADP

$$K_D = \frac{(K_{H1} + X)(X + K_{H3})}{X^2/K_{NH} + K_{H3}X/K_N}$$

DAN-ATP and DAN-ADP

$$K_D = \frac{(K_{H1} + X)(K_{H3} + X)}{X^2/K_{NH} + K_{H3}X/K_N}$$

DANSAMP and DAN-AMP

$$K_D = K_N(K_{H1} + X)(X/K_{H3} + 1)/X$$

The equations were input into the Pascal program. This allows calculation of K_D at different pH's with given parameters. For fitting the experimental data, each parameter was varied to obtain the best fitting curve.

REFERENCES

Bagshaw, C. R., & Trentham, D. R. (1974) *Biochem. J. 141*, 331−350.

Block, M. R., Lauquin, G. J. M., & Vignais, P. V. (1979) *FEBS Lett. 104*, 425−430.

Block, M. R., Lauquin, G. J. M., & Vignais, P. V. (1982) *Biochemistry 21*, 5451−5457.

Bock, R. M. (1960) in *The Enzymes 2*, 2nd ed., pp 3−38, Academic Press, New York.

Eckerskorn, T., & Klingenberg, M. (1987) *FEBS Lett. 226*, 166−170.

Ginsburg, A., Gorman, E. G., Neece, S. H., & Blackburn, M. B. (1987) *Biochemistry 26*, 5989−5996.

Flatmark, T., & Pederson, J. I. (1975) *Biochim. Biophys. Acta 416*, 53−103.

Hackenberg, H., Riccio, P., & Klingenberg, M. (1978) *Eur. J. Biochem. 88*, 373−378.

Himms-Hagen, J. (1976) *Annu. Rev. Physiol. 38*, 315−351.

Klingenberg, M. (1984) *Biochem. Soc. Trans. 12*, 390−393.

Klingenberg, M. (1988) *Biochemistry 27*, 781−791.

Klingenberg, M. (1990) *Trends Biochem. Sci. 15*, 108−112.

Klingenberg, M., & Winkler, E. (1985) *EMBO J. 4*, 3087−3092.

Klingenberg, M., Mayer, I., & Dahms, A. S. (1984) *Biochemistry 23*, 2442−2449.

Klingenberg, M., Mayer, I., & Appel, M. (1985) *Biochemistry 24*, 3650−3659.

Klingenberg, M., Herlt, M., & Winkler, E. (1986) *Methods Enzymol. 126*, 498−504.

Knirsch, M., Gawaz, M. P., & Klingenberg, M. (1989) *FEBS Lett. 244*, 427−432.

Lin, C. S., & Klingenberg, M. (1980) *FEBS Lett. 113*, 299−303.

Lin, C. S., & Klingenberg, M. (1982) *Biochemistry 21*, 2950−2956.

Mayer, I., Dahms, A. S., Riezler, W., & Klingenberg, M. (1984) *Biochemistry 23*, 2436−2442.

Nicholls, D. G. (1979) *Biochim. Biophys. Acta 549*, 1−29.

Ouellet, L., Laidler, K. J., & Morales, M. F. (1952) *Arch. Biochem. Biophys. 39*, 37−50.

Ross, P. D., & Subramanian, S. (1981) *Biochemistry 20*, 3096.

Scott, T. G., Spencer, R., Leonard, N., & Weber, G. (1970) *J. Am. Chem. Soc. 92*, 687.

Schimmel, P. (1993) *Science 259*, 1264−1265.

Smith, A. C. (1967) *Methods Enzymol. 10*, 81−86.

Smith, R. E., & Horwitz, B. A. (1969) *Physiol. Rev. 49*, 330−425.

Swenson, C. A., & Ritchie, P. A. (1979) *Biochemistry 18*, 3654−3658.

Wada, H., Nakamura, H., & Miyatake, K. (1977) *J. Carbohydr. Nucleosides Nucleotides 4*, 231−239.

Watanabe, T., Inoue, A., & Tonomura, Y. (1981) *J. Biochem. 90*, 957−965.

Weidemann, M., Erdelt, H., & Klingenberg, M. (1970) *Eur. J. Biochem. 16*, 313−335.

Winkler, E., & Klingenberg, M. (1992) *Eur. J. Biochem. 207*, 135−145.