The kinetics of nucleotide binding to the uncoupling protein (UCP) from brown adipose tissue mitochondria were studied with a filter binding method. Fast and slow phases of binding were observed, corresponding to the two-stage binding model based on equilibrium binding studies (Huang, S. G., and Klingenberg, M. (1996) Biochemistry 35, 7846–7854) (Reaction 1).

\[
\begin{align*}
K_d & \quad k_{\text{fast}} \\
U + N & \rightarrow UN & \rightarrow U^*N \\
& \quad k_{\text{slow}}
\end{align*}
\]

**REACTION 1**

Although this method determines total binding, only the slow phase can be resolved. The fast unresolved phase represents the formation of the initial loose UCP-nucleotide complex (UN; \(K_d \approx 2 \mu M\)), whereas the slow phase reflects the tight binding (U\(^*\)N) associated with a conformational change induced by the bound nucleotide. Best fits of the binding data yielded, for the slow phase, \(k_{-1}\) values of \(3.0 \times 10^{-3} s^{-1}\) for GTP, \(4.8 \times 10^{-3} s^{-1}\) for ATP, \(0.13 s^{-1}\) for GDP, and \(>0.7 s^{-1}\) for ADP and dissociation rate constants \(k_{-1}\) of \(0.10 \times 10^{-3} s^{-1}\) for GTP, \(0.58 \times 10^{-3} s^{-1}\) for ATP, \(8.8 \times 10^{-3} s^{-1}\) for GDP, and \(>0.3 s^{-1}\) for ADP at pH 6.7 and 4 °C. The rates were fairly pH- and temperature-dependent. The distribution constant \(K_{\text{d}} = (k_{\text{fast}}/k_{\text{slow}})\) between the tight and loose complexes ranged between 2 and 30, suggesting formation of 71–97% of the tight complex at equilibrium. The \(K_{\text{d}}\) decreases with increasing pH, indicating a progressively less tight complex population. Anions (SO\(_4\)\(^2-\)) form a loose complex with UCP, thus affecting the initial association step, but not the subsequent transition step. While the kinetic constants were verified by dilution and chase experiments as well as in mass action plots, they were further corroborated with data obtained by fluorescence competition measurements. Taken together, our results show that nucleotide binding to UCP occurs via a two-stage mechanism in which the initial loose complex rearranges slowly into a tight complex.

Nucleotide binding of the uncoupling protein (UCP)\(^1\) from brown adipose tissue mitochondria has been key in the isolation and characterization of this H\(^+\)-transporting protein (1, 2).

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\(^{3}\) The abbreviations used are: UCP, uncoupling protein; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; Mops, 3-(N-morpholino)propanesulfonic acid; Mes, 2-(N-morpholino)ethanesulfonic acid.

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By facilitating the transport of H\(^+\) into the mitochondrial matrix, UCP dissipates the electrochemical energy into heat, a function that has been related to the non-shivering thermogenesis of this specialized tissue (3–5). The uncoupling effect is regulated by long-chain fatty acids as activators and by purine nucleoside di- and triphosphates as inhibitors (reviewed in Refs. 6 and 7).

Several studies have shown that nucleotide binding to UCP exhibits a strong pH dependence (5, 8–10). The pH dependence has been attributed to the interplay of H\(^+\)-dissociating groups of both the protein and nucleotide. It was suggested that deprotonation of an acidic group (Asp/Glu) and of a putative His abolishes electrostatic interactions between the protein and nucleotide, whereas deprotonation at the terminal phosphate of the nucleotide enhances its electrostatic interaction with the protein (9, 10). Kinetic measurements with fluorescent dansylated nucleotides further show that protonation at Asp/Glu is a prerequisite for nucleotide binding, whereas nucleoside triphosphate binding requires formation of an additional positive charge (HisH\(^+\)) at the binding cleft (10).

Despite such progress in equilibrium binding studies, a further understanding of the mechanism of nucleotide binding requires more insight into the binding kinetics. In this work, we have employed a filter binding method to evaluate the slow kinetics of nucleotide binding. With this method, the sum of tight and loose binding was measured. These data were complemented by kinetic measurements using specific fluorescent nucleotide probes. The results were analyzed in terms of a two-stage binding model derived previously (11).

**EXPERIMENTAL PROCEDURES**

**Materials—**Nucleotides were purchased from Boehringer Mannheim and NEN Life Science Products. 2′-O-Dansylated nucleotides were synthesized as described (10). The Millipore Ultrafree-MC 30,000 NMWL filter unit was obtained from Millipore Corp. (Bedford, MA). Triton X-100 was from Sigma.

**Isolation of UCP—**UCP was isolated from a Triton X-100-soluble lysate of brown adipose tissue mitochondria according to the method of Lin and Klingenberg (2). The isolated UCP migrated as a single Coomasie Blue band at 33 kDa on SDS gel. Protein concentration was determined according to the method of Lowry et al. (12) using bovine serum albumin as a standard. Nucleotide-binding sites were assessed by \(^{32P}\)GTP binding with an anion-exchange method (13).

**Nucleotide Binding Measured by Filter Binding Method—**UCP (~300 µg/ml) and radiolabeled nucleotide (1.2–12 µM) were incubated in an Eppendorf cup for 0.5–180 min in a medium containing 12 mM Mops, 20 mM Na\(_2\)SO\(_4\), and 0.1 mM EDTA, pH 6.7, and 4 °C or as otherwise stated. At different time intervals, 100 µl of the mixture was withdrawn, applied to the filter, and centrifuged immediately for 1 min at 4 °C. 10–20 µl of filtrate was then taken for scintillation counting.

The binding data showed a biphasic time course of increase. The initial fast phase could not be resolved by this method, whereas the slow phase exhibited an exponential increase. In line with our previous results, we interpret the fast phase as formation of the initial loose UCP-nucleotide complex (UN) and the slow phase as formation of the tight complex (U\(^*\)N). Utilizing our previous two-stage binding model...
(11), a simplified reaction scheme is written as shown in Reaction 1 in the summary. \(K_d\) is an apparent dissociation constant in the presence of \(\text{Na}_2\text{SO}_4\), which was normally included (8). We assume that the rate-limiting step is the slow conformational change, and UN is formed so fast that a static equilibrium is maintained. Since the free nucleotide concentration was measured, the concentration of bound nucleotide, which is calculated as the concentration of total nucleotide minus the concentration of free nucleotide, represents the sum of the concentrations of both loose and tight complexes (UN + U*N).

The rate of U*N formation is written as shown in Equation 1.

\[
\frac{d(U*N)}{dt} = k_{21}(UN) - k_{-21}(U*N)
\]  

(Eq. 1)

Thus, Equation 2 follows,

\[
(U*N) = K_d(1 - e^{-\gamma})(UN) = a(UN)
\]  

(Eq. 2)

with \(K'_d = k_{-21}/k_{21}\).

Applying mass conservation, we have Equations 3 and 4.

\[
N = N + UN + U^*N = N(1 + U/K_d + aU/K_d)
\]  

(Eq. 3)

\[
U = U + UN + U^*N = U + U(1/K_d + aK_d)N
\]  

(Eq. 4)

Thus, \(N = N/(1 + U/K_d + aU/K_d)\). Substitution into Equation 4 and solving for \(U\), we have Equations 5–8.

\[
\sqrt{(N_o - U_o)(1 + a) + 2K_d(N_o + U_o)(1 + a) + K_d^2} - \sqrt{(N_o - U_o)(1 + a) - K_d} = \frac{U}{2(1 + a)}
\]  

(Eq. 5)

\[
UN = (U_o - U_o)(1 + a)
\]  

(Eq. 6)

\[
U*N = a(U_o - U_o)(1 + a)
\]  

(Eq. 7)

\[
UN + U^*N = U_o - U
\]  

(Eq. 8)

For the mass action plot, the concentration of bound nucleotide is calculated as shown in Equation 9.

\[
\text{Bound} = (UN + U^*N)/(U_o - U)
\]  

(Eq. 9)

The concentration of free nucleotide (N) is obtained from Equations 3 and 5. The x axis in the mass action plot (Bound/free) is thus calculated as shown in Equation 10.

\[
\text{Bound/free} = (UN + U^*N)/(GTP)_{ex}
\]  

(Eq. 10)

Least-square fitting was performed with the MicroCal Origin program (Version 2.75, MicroCal Software Inc.). Computation of the theoretical curves was achieved with a Turbo Pascal program (Version 6).

Fig. 1 shows the time progress of nucleotide binding to isolated UCP. To obtain rates, a low concentration of GTP (1.2 \(\mu\)M) and 20 mM \(\text{Na}_2\text{SO}_4\), which competes for GTP binding, were added (10). In all measurements, binding data showed a biphasic fast and slow increase. The time required for half-maximal binding was ~17 min (Fig. 1A). Similarly, ATP binding was slow (Fig. 1C). However, the nucleoside diphosphates GDP (Fig. 1B) and ADP (Fig. 1D) bound much faster.

The measured speeds (Fig. 1) are best fitted (solid lines) according to the two-stage binding model. To estimate the dissociation constant (\(K_d\)) for the initial loose complex, we made use of the value measured at 0.5 min. For example, the concentration of bound GTP measured at 0.5 min (the first point) was ~0.11 \(\mu\)M, corresponding to a \(K_d\) of 21 \(\mu\)M. The calculated \(K'_d\) values varied only between 12 and 28 \(\mu\)M from various measurements. Since these are rough estimates, we fixed \(K'_d\) at an average value of 14 \(\mu\)M and performed least-square fitting for the two parameters \(K'_d\) and \(k_{-21}\) according to Equation 8. Meaningful fitting can be obtained for the slow binding process measured at 1.2 \(\mu\)M. Thus, for GTP binding (Fig. 1A), the best fit revealed very slow transition rates and a ratio of tight to loose complex of 30:1 (Table I). GTP binding at 12 \(\mu\)M was much faster (Fig. 1A, inset) and could be fitted only with uncertainty. Using the parameters obtained for [GTP] = 1.2 \(\mu\)M (Table I), the theoretical binding calculated for [GTP] = 12 \(\mu\)M fit the experimental data quite well. Using the same procedure, we extracted the kinetic parameters for GDP (Fig. 1B) and ATP (Fig. 1C). Since the transition was faster with ADP (Fig. 1D), only a meaningful distribution constant (\(K'_d\)) could be obtained, and lower limits were estimated for the transition rates. The overall dissociation constants (\(K_d\)) calculated from the extracted parameters (Table I) are in full agreement with our previous data measured by equilibrium dialysis (8), on anion-exchange method (9), and fluorescence titrations (10).

At higher pH (Fig. 2A), GTP binding appeared to be faster, but weaker with a drastic decrease in the tight complex population (Table I). We also compared GTP binding obtained at 4 and 14.5 °C to illustrate the strong temperature dependence.
A best fit for the data points at 14.5 °C required $K_c = 25.3$ and $k_{-1} = 1.0 \times 10^{-3}$ s$^{-1}$. The data suggest that temperature did not affect the tight/loose complex distribution ($K_c$), but the transition rates at 14.5 °C were nearly 10-fold faster than at 4 °C.

To further evaluate the time-dependent nucleotide binding, we studied the nucleotide concentration dependence. Fig. 3 summarizes in mass action plots the GTP binding data measured after incubation for 3, 20, and 180 min. We calculated the concentrations of bound (UN + U*N) and free GTP according to Equation 9 using the parameters given in Table I. For pH 6.7 (Fig. 3A), the calculated mass action plots (solid lines) are straight lines of varying slopes, with $K_D = 12.9, 3.8, \text{ and } 1.0 \mu M$ for 3, 20, and 180 min, respectively. At pH 7.4 (Fig. 3B), GTP binding was weaker, with $K_D$ values 2–4-fold higher than at pH 6.7. GTP binding at pH 7.8 was rather weak, with 3–16-fold higher $K_D$ values. In all cases, the theoretical calculations give good fits to the data points, although the latter scattered.

**Table I**

Kinetic parameters of slow-phase nucleotide binding to isolated UCP

|            | GTP         | GDP, pH 6.7 | ATP, pH 6.7 | ADP, pH 6.7 |
|------------|-------------|-------------|-------------|-------------|
| $k_{-1}$ ($\times 10^4$ s$^{-1}$) | pH 6.7: 3.0 ± 1.0 | 1.5 ± 0.2 | >2.1 | 130 ± 30 | 4.8 ± 0.4 | >700 |
| $k_{-1}$ ($\times 10^3$ s$^{-1}$) | pH 7.4: 0.10 ± 0.03 | 0.53 ± 0.08 | >3.0 | 8.8 ± 2.2 | 0.58 ± 0.05 | >300 |
| $K_c$ $i$  | pH 7.8: 30.2 ± 7.2 | 2.8 ± 0.2 | 0.7 ± 0.2 | 14.5 ± 1.2 | 8.2 ± 0.3 | 2.4 |
| $K_D$ ($\mu M$) | pH 6.7: 0.45 | 3.7 | 8.2 | 0.90 | 1.52 | 4 |
|            | pH 7.4: 3.0  | 6.1 | 1.5 | 0.2  | 0.58 | 0.4 |
|            | pH 7.8: 3.0  | 6.1 | 1.5 | 0.2  | 0.58 | 0.4 |

**Fig. 2.** GTP binding to isolated UCP is dependent on pH and temperature. 2.2 $\mu M$ UCP was incubated with 1.2 $\mu M$ [14C]GTP at pH 6.7, 7.4, and 7.8 and 4 °C (A) and at pH 6.7 and 4 °C and 14.5 °C.

**Fig. 3.** Mass action plots of GTP binding to isolated UCP. UCP (8–10 $\mu M$) was incubated with 0.5–15 $\mu M$ [14C]GTP at the indicated pH values and 4 °C for 3, 20, and 180 min. The solid lines were computed according to Equation 9.
strongly at pH 7.8 (Fig. 3C).

To verify the validity of the filtration method, we employed two approaches to measure the dissociation rate. First, UCP was incubated (23 °C) with [14C]GTP until equilibrium was reached, and excess unlabelled GTP was added to displace the prebound [14C]GTP. As shown in Fig. 4A, the concentration of prebound [14C]GTP decreased exponentially. Best fits of the data for pH 6.7 and 7.4 yielded \( k_{-1} = 0.44 \times 10^{-3} \) and \( 1.5 \times 10^{-3} \) s\(^{-1}\), respectively. The time course of free [14C]GTP released into the supernatant followed an exponential increase with rate constants of 0.98 \( \times 10^{-3} \) and \( 3.6 \times 10^{-3} \) s\(^{-1}\), respectively (Fig. 4B). The rapid drop in the complex concentration upon dilution revealed the presence of a fast dissociating loose complex (<18%), and a tight complex (>82%), which gave the slow phase of dissociation with a rate constant of \( 1.1 \times 10^{-3} \) s\(^{-1}\), in fair agreement with the rate determined above by displacement with excess unlabelled GTP.

Anions were known previously to be competitive inhibitors of nucleotide binding (8, 10). Here we studied the effects of anions on the two complexes. The influence of anion on nucleotide binding and dissociation is illustrated with Na\(_2\)SO\(_4\). With increasing [SO\(_4^{2-}\)] in the incubation media, the GTP binding curve was shifted progressively downward (Fig. 5A). The anion effect can be explained in that SO\(_4^{2-}\) can competitively bind to the nucleotide-binding site and increase the \( K_d \) for the initial loose complex (10). Theoretical fitting using data of Table I gave \( K_d \) values of 3.8, 9.4, and 32.7 \( \mu \)M at 2.1, 10, and 48 mM SO\(_4^{2-}\), respectively. Attempts to fit the data with constant \( K_d \) but varying \( K'_{d} \) or \( k_{-1} \), failed, suggesting that SO\(_4^{2-}\) does not influence the subsequent conformational change step. Such a finding was also reported for ATP or ADP binding to myosin (16). A plot of \( K_d \) against [SO\(_4^{2-}\)] gives a straight line (Fig. 5A, inset), yielding an intrinsic dissociation constant (\( K'_{d} \)) in the absence of any anions of 25.5 ± 0.6 \( \mu \)M and an SO\(_4^{2-}\) inhibition constant \( (K_{i}^{SO4^{2-}}) \) of 4.0 mM. The SO\(_4^{2-}\) inhibition constant measured with this method is close to the value (1.0 mM) determined previously by fluorescence titrations (10). Indeed, when [14C]GTP dissociation induced by excess unlabelled GTP was followed in the presence of SO\(_4^{2-}\) (Fig. 5B), little difference was observed in the dissociation rate with 2.1 or 48 mM Na\(_2\)SO\(_4\).

We also measured GTP binding to intact mitochondria from brown adipose tissue with the filter binding method in parallel with the centrifugation method (data not shown). The two measurements yielded the same results. Best fits of the binding data revealed \( K'_{d} = 123 \), suggesting 4-fold higher tight complex formation with UCP in the mitochondria than in the isolated state. While at 4 °C the transition rate \( (k_{-1}) \) was comparable to that for the isolated protein, the dissociation rate \( (k_{-1} = 0.033 \times 10^{-3} \) s\(^{-1}\) \) was nearly 3-fold slower. The transition rates were 8-fold faster at 23 °C than at 4 °C, similar to the data with the isolated protein.

**Kinetics of Nucleotide Binding Measured by Fluorescence Methods**—The slow-phase kinetics were also measured by competition with fluorescent nucleotides as follows. Dansyl-ADP was shown previously to bind to and dissociate from UCP rapidly (10). The displacement of prebound dansyl-ADP by nucleotides was used to record their transition rates (see "Experimental Procedures"). When experiments were performed with various GTP concentrations (1–8 \( \mu \)M), the fluorescence decrease could be fit with the same rate constant, suggesting that rate limitation indeed applies. The rate of fluorescence decrease depends on the added nucleotide (Fig. 6). While GTP and ATP caused a slow fluorescence increase, GDP and ADP elicited a faster fluorescence response, with ADP being even faster than GDP. The decreases in fluorescence can be well fit (solid lines) with a single exponential equation (Table II).

To measure the dissociation rate of the tight U\(^{+}\)N complex, excess dansyl-GTP was added to the preformed UCP-nucleotide complex. As shown in Fig. 7, the rate of fluorescence increase depends on the competing nucleotide species. It was slow with GTP and ATP, but faster with GDP and ADP. The rate constants \( (k_{-1}) \) were obtained by fitting and are given in Table II.
Here we studied the time course of the total binding by a filter binding method that revealed a clearly biphasic binding process. Although the initial fast phase of binding could not be resolved with this method, we assume that the rate would be the same ($k_{on} = 3.6 \times 10^6 \text{ M}^{-1} \text{s}^{-1}$) as previously determined for the dansylated nucleotides by a stopped-flow method (10). The further slow increase in binding was attributed to the subsequent transition to the tight complex. The existence of loose and tight complexes was also demonstrated in dilution experiments (Fig. 4B). The biphasic kinetics can be best described by the two-stage binding model. Furthermore, the time-dependent change of the mass action plots could be quantitatively fitted by this model, thus providing experimental support in two dimensions of time and GTP concentration.

The two-stage time course of binding is simulated by theoretical computations to further illustrate the biphasic binding model. For this purpose, we fixed the dissociation constant for the initial loose complex ($K_d = 14 \mu M$) and the tight-to-loose transition rate constant ($k_{-1} = 0.1 \times 10^{-3} \text{ s}^{-1}$) (Table I), but varied the tight/loose distribution constant ($K'_c$) (Fig. 8A). The total binding ($\text{UN} + \text{U*N}$) is clearly very sensitive to $K'_c$, as was shown by the pH dependence (Fig. 2A). Increasing the $K_d$ at a constant $K'_c$ shifts the binding curve downward without altering the curvature (Fig. 2B), in line with the effects of anions (Fig. 5). An increase in the transition rates ($k_{+1}$ and $k_{-1}$) enhances the curvature without affecting the end point (Fig. 8C), in agreement with the faster binding at elevated temperatures (Fig. 2B). The sensitivity of these parameters inherent to the two-stage binding model has allowed an unambiguous extraction of the kinetic parameters. In Fig. 8D, we calculated the time course of the various UCP-binding species using the parameters given in Table I. The free UCP concentration ($U$) decreases exponentially as binding proceeds. The loose complex ($\text{UN}$) forms instantly (here $0.5 \mu M$ at time 0), but its concentration decreases as the conformational change occurs. The increase in the tight complex ($\text{U*N}$) follows an exponential progression course from time 0. Since the filter binding method measures both the loose and tight complexes, the observed binding ($\text{UN} + \text{U*N}$) increases exponentially from $0.5 \mu M$, thus showing a biphasic time course of nucleotide binding.

As shown in Table I, the tight/loose distribution varies moderately among the four nucleotides so that at equilibrium (pH 6.7 and 4°C), the tight complex ($\text{U*N}$) accounts for 71–97% of the total UCP-nucleotide complex. With increasing pH, the transition rate constant ($k_{+1}$) for GTP varied only 2-fold; whereas the dissociation rate constant ($k_{-1}$) increased 5-fold at pH 7.4 and nearly 30-fold at pH 7.8. The $K'_c$ decreased by $-10^{-}$

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**Table II**

| Nucleotide | GTP | GDP | ATP | ADP |
|------------|-----|-----|-----|-----|
| $k_{+1}$ ($\times 10^3 \text{ s}^{-1}$) | 16.8 ± 0.2 | 66.3 ± 0.7 | 25.6 ± 0.6 | 143 ± 5 |
| $k_{-1}$ ($\times 10^3 \text{ s}^{-1}$) | 2.50 ± 0.001 | 11.2 ± 0.1 | 4.06 ± 0.002 | 21.9 ± 0.8 |
| $K'_c$ | 6.8 | 5.9 | 6.4 | 6.8 |

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**DISCUSSION**

Nucleotide binding to UCP was found to follow a two-stage process, with an initial fast but loose binding state and a slow transition into a tight binding state. In our previous equilibrium experiments (10), the loose and tight complexes were verified by comparing the binding data measured by an anion-exchange method and fluorescence titrations. Most important, the inhibition of $\text{H}^+$ transport was shown to require the tight binding state. Furthermore, the increased resistance to tryptic digestion of the tight complex witnesses that a major conformational change is involved (11).
The dissociation of prebound nucleotide from these enzymes was reported to be rather slow. The rate constants for dissociation of ADP and ATP from G-actin were 3.3 × 10^{-3} and 5 × 10^{-4} s^{-1}, respectively (20). ADP dissociation from the platelet ADP receptor was found to be 2.4 × 10^{-3} s^{-1} (21). The dissociation rate in myosin was very temperature-sensitive (22), increasing from 0.07 to 1.4 s^{-1} as the temperature increased from 5 to 21 °C. The G-protein family also had a high affinity for GTP and GDP. The nucleotides dissociate from the Ras-related nucleol G-protein slowly, with rate constants of 1.2 × 10^{-5} and 1.4 × 10^{-5} s^{-1}, respectively (23, 24). Slow dissociation (0.85 × 10^{-3} s^{-1} for GDP and 5 × 10^{-3} s^{-1} for GTP) was observed also for elongation factor Tu from *Thermus thermophilus* (18). The slow dissociation in these proteins may suggest a common occluded state of nucleotide in the binding pocket following conformational rearrangement in the protein.

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