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Subunit–subunit interactions in TF_1 as revealed by ligand binding to isolated and integrated α and β subunits *

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The binding of 3′-*O*-(1-naphthoyl)adenosinetriphosphate (1-naphthoyl-ATP), ATP and ADP to TF_1 and to the isolated α and β subunits was investigated by measuring changes of intrinsic protein fluorescence and of fluorescence anisotropy of 1-naphthoyl-ATP upon binding. The following results were obtained. (1) The isolated α and β subunits bind 1 mol 1-naphthoyl-ATP with a dissociation constant (K_D(1-naphthoyl-ATP)) of 4.6 μM and 1.9 μM, respectively. (2) The K_D(ATP) for α and β subunits is 8 μM and 11 μM, respectively. (3) The K_D(ADP) for α and β subunits is 38 μM and 7 μM, respectively. (4) TF_1 binds 2 mol 1-naphthoyl-ATP per mol enzyme with $K_D = 170$ nM. (5) The rate constant for 1-naphthoyl-ATP binding to α and β subunit is more than $5 \cdot 10^4$ $M^{-1}s^{-1}$. (6) The rate constant for 1-naphthoyl-ATP binding to TF_1 is $6.6 \cdot 10^3$ $M^{-1} \cdot s^{-1}$ (monophasic reaction); the rate constant for its dissociation in the presence of ATP is biphasic with a fast first phase ($k^A_{-1} = 3 \cdot 10^{-3}$ s^{-1}) and a slower second phase ($k^A_{-2} < 0.2 \cdot 10^{-3}$ s^{-1}). From the appearance of a second peak in the fluorescence emission spectrum of 1-naphthoyl-ATP upon binding it is concluded that the binding sites in TF_1 are located in an environment more hydrophobic than the binding sites on isolated α and β subunits. The differences in kinetic and thermodynamic parameters for ligand binding to isolated versus integrated α and β subunits, respectively, are explained by interactions between these subunits in the enzyme complex.

Introduction

Membrane-bound ATPases of the F_0F_1-type have been isolated from various organelles and organisms. Driven by a transmembrane proton gradient, they promote formation of ATP from ADP and P_i in a reversible reaction. The detailed mechanism by which proton translocation is coupled to synthesis of ATP is still a matter of dispute [1–5].

F_0F_1-ATPases are oligomeric enzymes consisting of at least eight different types of subunit. While the β subunits – maybe in collaboration with the α subunits – are supposed by a majority of authors to form the catalytic sites, discussion now centres on the significance of subunit–subunit interactions which have been proposed as an underlying principle in F_1-catalysed ATP-synthesis [6,7].

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Abbreviations: TF_1, catalytic part of the ATP-synthase/hydrolase from the thermophilic bacterium PS3; 1-naphthoyl-ATP, 3′-*O*-(1-naphthoyl)adenosinetriphosphate.

We studied this problem by comparing the nucleotide binding behaviour of isolated native subunits and of F_1-integrated subunits, respectively. Hence, this approach requires the investigation of functionally viable subunits. The ATPase from the thermophilic bacterium PS3, TF_0F_1, was chosen for these studies because of its remarkable stability. After dissociation by urea, an active complex can be reconstituted from the isolated subunits [8]. An additional advantage of this ATPase lies in the fact that it is completely devoid of tightly bound endogenous nucleotides [9]. The enzyme can therefore be directly assayed for its nucleitde binding capability; in the study presented here, this was achieved by use of the fluorescent ATP-analogue 3'-*O*-(1-naphthoyl)adenosinetriphosphate (1-naphthoyl-ATP). By measuring fluorescence changes of F_1 and changes of fluorescence anisotropy of 1-naphthoyl-ADP and 1-naphthoyl-ATP, it has been shown earlier [10] that these analogues are bound to mitochondrial F_1; they also competitively inhibit ATP-synthesis and -hydrolysis [11]. In this paper we describe binding properties of both TF_1 and the isolated α and β subunits, respectively, towards these analogues. While both the α and the β subunits are able to bind 1 mol 1-naphthoyl-ATP per mol of the respective subunit, the maximum number of analogue molecules bound per molecule of TF_1 is only two. These results corroborate the conjecture of subunit–subunit interactions in F_1-type ATPases.

Materials and Methods

Preparation of TF_1-ATPase and subunits

Cells of the thermophilic bacterium PS3 were supplied by Drs. Y. Kagawa and M. Yoshida (Jichi Medical School, Minamikawachi-Machi, Japan). TF_1 and subunits were prepared according to Ref. 12 with minor modifications: (1) the treatment with guanidine hydrochlorid in the preparation of subunits was omitted; (2) a DEAE-Sephacel column was used instead of a Whatman DE-52 column. Purified TF_1 was lyophilized from water, while all subunits were lyophilized from 10 mM Tris buffer (pH 8.0), with 10–15% sucrose (in the case of the α subunit aggregation was prevented by addition of 0.1 mM dithiothreitol.). TF_1 and the isolated α and β subunit were stored in lyophilized state at −80°C.

High-performance liquid chromatography (HPLC)

The purity of the preparations of TF_1 and the isolated α and β subunit was checked by HPLC, performed on a Waters apparatus (injector U6K, pump 510, and detector M481; Waters Associated Inc., Milford, MA), by size exclusion chromatography in the presence of sodium dodecylsulfate (column TSK G 3000 SW from Toyo Soda, Flow rate 1 ml/min). The elution buffer (0.1 M sodium phosphate (pH 6.0)/0.1% sodium dodecylsulfate) was filtered through a 0.45 μm filter (type HA), placed in a sintered glass vacuum filter (both from Millipore, U.S.A.), and degassed by sonication prior to use. The elution profile was monitored at 220 nm. All preparations were free from contaminating proteins.

Fluorescence techniques

The fluorescence measurements were carried out with a spectrofluorometer type SLM 4800 S. Fluorescence intensities (including fluorescence spectra) were measured using a reference channel to correct for time-dependent and wavelength-dependent fluctuations of the exciting light. Fluorescence spectra of sample and solvent were recorded separately and stored in a computer (Hewlett Packard 9825B) to allow for correction of solvent effects. All shown fluorescence spectra are difference spectra of sample spectrum minus solvent spectrum. Inner filter effects in titration experiments were corrected for as described in Ref. 10. For measurements of fluorescence anisotropy, the set-up of the instrument was changed according to Ref. 13.

Fluorescence titrations were carried out in a buffer containing 50 mM Tris-HCl (pH 8.0)/50 mM KCl/2.5 mM $MgCl_2$ (α subunit: +1 mM dithiothreitol). The buffer solution was filtered through a unipore polycarbonate membrane (Bio-Rad, 0.4 μm pore size) prior to use. Fluorescence was measured after the association reaction was completed, i.e., the state of equilibrium had been attained as judged by the time dependence of the fluorescence signal. All experiments were performed at 45°C; see Ref. 9 for the temperature dependence of different functions of TF_1. Titra-

tion experiments were evaluated by a computer-supported nonlinear least-square fit [14]. In the case of multiple binding sites (binding of 1-naphthoyl-ATP towards TF_1), the assumption of a model with identical and independent sites was sufficient to obtain an optimal fit.

Chemicals and General methods

Sodium dodecyl sulfate and L-tyrosine were purchased from Serva (Heidelberg). Boehringer (Mannheim) supplied ADP, ATP, NADH, phospho*enol*pyruvate, and the enzymes pyruvate kinase and lactate dehydrogenase. 1-naphthoyl-ATP was synthesized as described in Ref. 15. Its purity was checked by thin-layer chromatography on cellulose plates (Merck, Darmstadt) [15]. The concentration of 1-naphthoyl-ATP was determined spectrophotometrically ($\varepsilon^{259\,nm}_{1\text{-naphthoyl-ATP}} = 16\,000\ M^{-1} \cdot cm^{-1}$ [16]). Protein concentrations were determined using the method of Lowry et al. [17] with bovine serum albumin as a standard. In addition, the TF_1-concentration was determined spectroscopically ($A^{1\,mg/ml}_{278\,nm} = 0.5$) [18]; the molecular mass of TF_1 was taken to be 380 000 [18].

ATPase-activity (one unit is defined as μmol ATP hydrolyzed $\cdot\ min^{-1}$ at 30°C) was measured either in an ATP-regenerating test system [19] or by colorimetrical determination of the released inorganic phosphate [20]. The specific activity of various preparations of TF_1 fell between 10 and 20 U/mg; however, these variations of the specific activity were insignificant as to nucleotide-binding properties.

Results

Intrinsic fluorescence and 1-naphthoyl-ATP fluorescence

Fig. 1 shows the intrinsic fluorescence emission spectrum of nucleotide free TF_1 with a distinct peak at 304 nm and a subtle shoulder at 350–360 nm (curve 1). The shape of the bands and the position of peak and shoulder suggest that tyr-residues cause the peak at 304 nm, while trp-residues are responsible for the subtle shoulder at 350–360 nm. This is consistent with the amino acid analysis of TF_1 [18] which has shown that TF_1 contains both tyr- and trp-residues.

Binding of 1-naphthoyl-ATP to the enzyme results in changes of the fluorescence spectrum (Fig. 1): as can be seen from curve 3, this is not a pure superposition of the spectra of TF_1 (curve 1) and of 1-naphthoyl-ATP (curve 2). The most intriguing change is the appearance of a second peak in the emission spectrum of 1-naphthoyl-ATP with a maximum at 365 nm. This is even more obvious when the difference between curve 3 and 2 is plotted (Fig. 1, bottom). In model experiments with 1-naphthoyl-ATP in water/dioxane mixtures, the same peak appeared when the polarity of the solvent was decreased (Weber, J., Rögner, M. and Schäfer, G., unpublished data). The height of this peak depends on the ratio of concentrations of TF_1 to 1-naphthoyl-ATP; the spectrum at saturation is shown in Fig. 2a. The appearance of this peak is presumably due to a shift of the fluorescence band of 1-naphthoyl-ATP which occurs upon binding to a relatively hydrophobic site.

Parallel to the increase of the 1-naphthoyl-ATP-fluorescence at 365 nm, the tyr-fluorescence

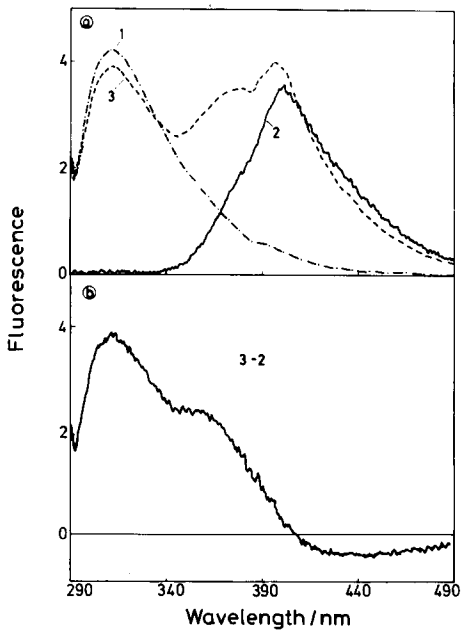


Fig. 1. Intrinsic fluorescence spectrum of TF_1 without and with 1-naphthoyl-ATP (N-ATP): (a) fluorescence spectrum F(TF_1) (0.66 μM) (curve 1), F(1-naphthoyl-ATP) (1.32 μM) (curve 2) and F(TF_1 + 1-naphthoyl-ATP) (λ_{ex} = 285 nm) (curve 3); (b) fluorescence difference spectrum F(TF_1 + 1-naphthoyl-ATP)-F(1-naphthoyl-ATP).

at 304 nm is quenched: at saturation with 1-naphthoyl-ATP, the decrease is 17%. Binding of ADP and ATP results in a quench of the tyr-fluorescence as well; however, the decrease at saturation is less than 5%.

At 360 nm no increase can be detected for the β subunit (Fig. 2c), and only a small increase can be seen in the case of the α subunit (Fig. 2b). If the fluorescence is excited at 293 nm, the fluorescence emission at 360 nm is increased strongly with TF_1, whereas the α and the β subunit show no increase at all (data not shown). Given that the described binding sites on the isolated subunits and on the holoenzyme are identical, these results may be taken as evidence for an increased hydrophobicity of the respective sites due to subunit–subunit interactions.

Equilibrium binding of 1-naphthoyl-ATP to TF_1

The decrease of the tyrosine fluorescence (304 nm) can be used for studying the binding of 1-naphthoyl-ATP to TF_1 as has been shown in similar experiments with mitochondrial F_1 [10]. Data from Fig. 2a, corrected for inner filter effects, and analogous measurements at different concentrations of 1-naphthoyl-ATP have been used for such titration experiments (Fig. 3a). An optimal fit of a theoretical curve to the data yields two binding sites for 1-naphthoyl-ATP per TF_1. Although the fluorescence increase at 365 nm is higher than the simultaneous decrease at 304 nm, this increase cannot be used for a quantitative evaluation. At 365 nm, two effects are superimposed: (a) an increase of the 1-naphthoyl-ATP fluorescence due to the shift of its fluorescence spectrum, and (b) a decrease of the intrinsic fluorescence of the protein. A separation of both effects and a correction for inner filter effects is practically impossible at this wavelength.

As shown in Ref. 10, also the polarization of the fluorescent ligand 1-naphthoyl-ATP can be used for a titration of the binding sites; the anisotropy is directly proportional to the degree of saturation. Free 1-naphthoyl-ATP shows an anisotropy of 0.011, while for 1-naphthoyl-ATP bound to TF_1-ATPase the anisotropy is 0.21 due to the restriction of rotational motion upon binding to the macromolecule. Fig. 3b shows the fluorescence anisotropy of 1-naphthoyl-ATP as a function of TF_1 concentration. From the intersection of the extrapolated linear parts of the graph a number of

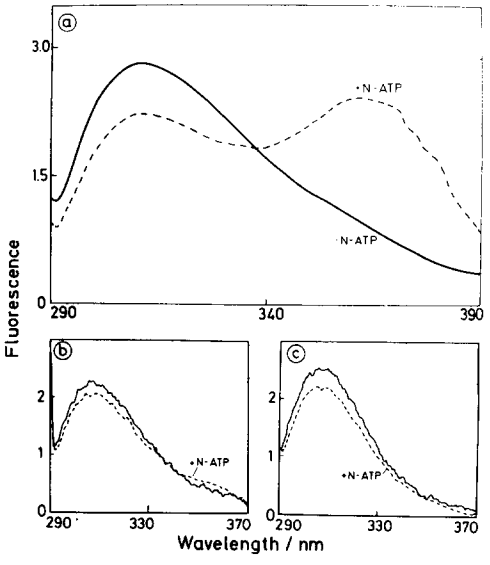


Fig. 2. Intrinsic fluorescence of TF_1 (0.66 μM), α subunit and β subunit (each 4 μM) (1-naphthoyl-ATP (N-ATP), ± 4 μM; λ_{ex}, 285 nm): (a) Fluorescence spectrum of TF_1 and fluorescence difference spectrum ($F(TF_1 + 1\text{-naphthoyl-ATP}) - F(1\text{-naphthoyl-ATP})$) recorded at saturation conditions. (b) and (c) Fluorescence spectra (solid lines) and fluorescence difference spectra ($F(\alpha/\beta + 1\text{-naphthoyl-ATP}) - F(1\text{-naphthoyl-ATP})$, dashed lines) of isolated α (b) and β (c) subunits.

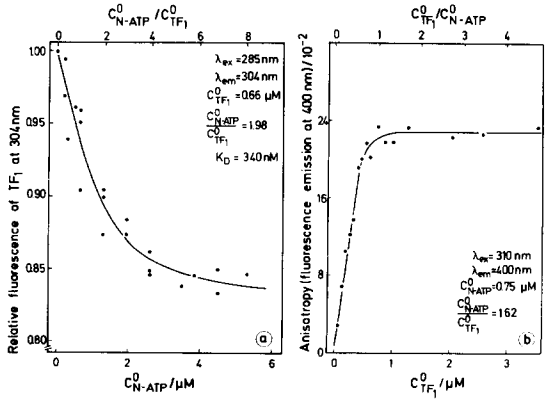


Fig. 3. (a) Stoichiometric fluorescence titration of 1-naphthoyl-ATP with TF_1: relative intrinsic TF_1 fluorescence at 304 nm as a function of the 1-naphthoyl-ATP concentration (bottom) and the 1-naphthoyl-ATP/TF_1 ratio (top). (b) Stoichiometric fluorescence titration of 1-naphthoyl-ATP with TF_1: fluorescence anisotropy of 1-naphthoyl-ATP as a function of TF_1 concentration (bottom) and the TF_1/1-naphthoyl-ATP ratio (top).

two binding sites per TF_1 (exact value for N: 1.62) can be calculated. This value is in fair agreement with the corresponding result obtained by observation of the protein fluorescence. Moreover, it should be pointed out that the same stoichiometry was obtained with the analogous enzyme from bovine heart mitochondria [10].

Both fluorescence techniques described above for the determination of the number of binding sites were also used to measure the equilibrium constant(s) for the interaction of TF_1 with 1-naphthoyl-ATP. From the curve presented in Fig. 3a) (quench of protein fluorescence upon titration with 1-naphthoyl-ATP), K_D was calculated to be 340 nM. Titrations evaluating the anisotropy of 1-naphthoyl-ATP upon addition of TF_1 resulted in a dissociation constant of 170 nM (Fig. 4); this latter value was used for all further calculations (e.g., kinetic constants) because of its higher reliability. Error factors for both values are given in Table I; the K_D of 170 nM lies even within the margin of error of the 340 nM value. As a consequence, titration experiments indicate that 1-naphthoyl-ATP binds to two identical binding sites on TF_1 with a dissociation constant of 170 nM.

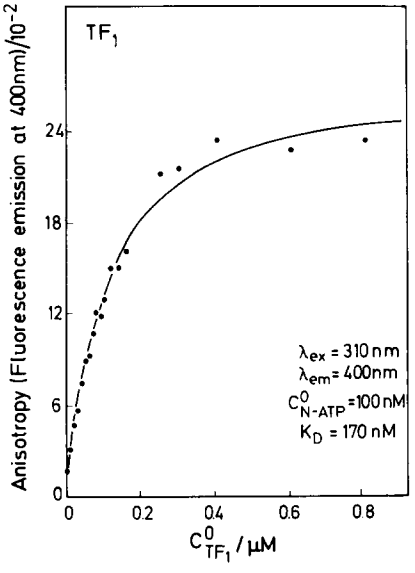


Fig. 4. Fluorescence titration of 1-naphthoyl-ATP with TF_1. Fluorescence anisotropy of 1-naphthoyl-ATP as a function of the concentration of TF_1; anisotropy at saturation is 0.21 ± 0.01. Calculation based on the assumption of two binding sites (see Fig. 3).

Equilibrium binding of 1-naphthoyl-ATP to the isolated α and β subunits

The tyr-fluorescence of both of the subunits is quenched upon titration with 1-naphthoyl-ATP (see Fig. 2b and c); however, the quench at saturation was found to be less than 5% for α as well as for β, a value too small to be used for precise titration experiments. Therefore, the binding properties of 1-naphthoyl-ATP towards the isolated subunits were determined only by measuring the fluorescence anisotropy of 1-naphthoyl-ATP. Fig. 5 shows the anisotropy of 1-naphthoyl-ATP as a function of the addition of α and β subunits. Due to the lower molecular mass of isolated subunits, the rotational mobility of bound ligand is less restricted as compared to native TF_1; this results in a lower anisotropy upon saturation. The exact evaluation (N and K_D) of both titration curves is summarized in Table I. Taking into account the experimental error, there is one binding site for 1-naphthoyl-ATP on each of the large subunits (α and β); compared with the holoenzyme, the affinities of these binding sites towards 1-naphthoyl-ATP are reduced by a factor of more than 10.

Kinetics of 1-naphthoyl-ATP binding and release

Kinetics of 1-naphthoyl-ATP binding and release from TF_1 were measured via the time-course of the fluorescence anisotropy of 1-naphthoyl-ATP.

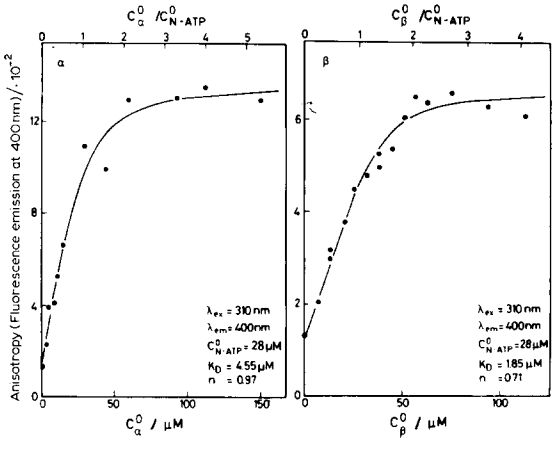


Fig. 5. Fluorescence titration of 1-naphthoyl-ATP with α and β subunit: fluorescence anisotropy of 1-naphthoyl-ATP as a function of the concentration of isolated α (left) and β (right) subunit, respectively. Anisotropy at saturation is 0.13 ± 0.005 for α and 0.065 ± 0.005 for β subunit (data not shown).

An example for the binding of the nucleotide to the enzyme is depicted in Fig. 6a). Although a slight systematic deviation can be seen, the assumption of a second-order-rate law appears to be correct. Evaluated on basis of this assumption (Fig. 6c), the rate constant was found to be $k_1 = 6.6 \cdot 10^3\ M^{-1} \cdot s^{-1}$ (average of seven measurements).

Fig. 6b shows the displacement of 1-naphthoyl-ATP from its binding sites upon addition of an excess amount of ATP (more than 100-fold). The time-course of the release reaction, treated as a pseudo-first-order, turns out to be biphasic (Fig. 6d). Obviously, the release of 1-naphthoyl-ATP from the two binding sites after addition of ATP does not occur with identical rates. The first phase represents the superposition of a fast and a slow release reaction; after complete displacement of 1-naphthoyl-ATP from the first site (fast rate), the slow release from the second site is still going on. The rate constants were calculated to be $k^A_{-1} = 2.6 \cdot 10^{-3}\ s^{-1}$ and $k^A_{-2} < 0.2 \cdot 10^{-3}\ s^{-1}$ (the super-

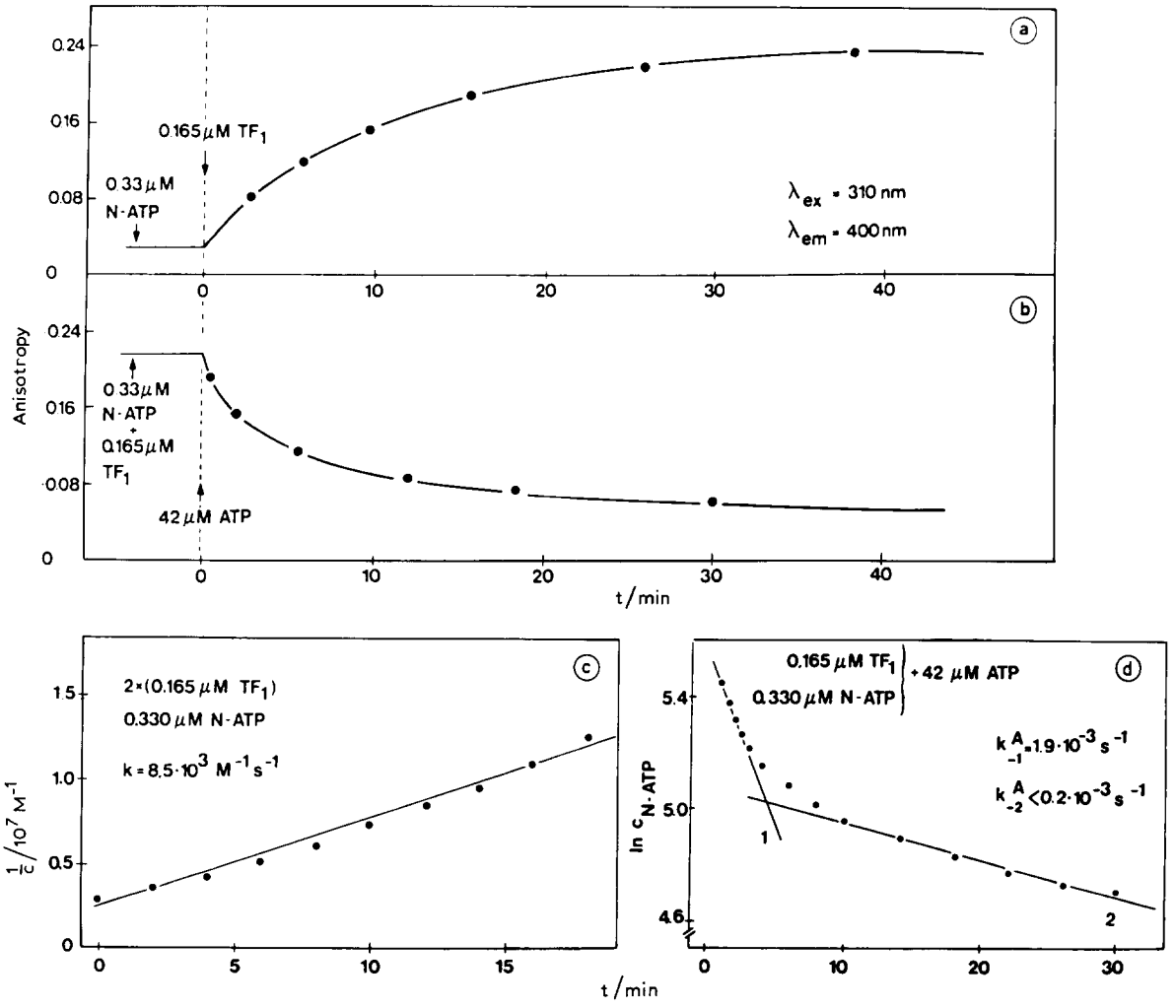


Fig. 6. (a/b) Kinetics of 1-naphthoyl-ATP binding to and release from TF_1: fluorescence anisotropy of 1-naphthoyl-ATP at 400 nm was measured as a function of (a) binding of 1-naphthoyl-ATP to the enzyme, and (b) release of 1-naphthoyl-ATP upon addition of excess ATP. (c) Kinetics of 1-naphthoyl-ATP binding to TF_1: reciprocal plot of the data from Fig. 6a; the second-order rate constant has been calculated on the basis of two binding sites on TF_1. (d) Kinetics of 1-naphthoyl-ATP release from TF_1 by addition of ATP: Semi-logarithmic plot of the data from Fig. 6b, showing biphasic pseudo-first-order kinetics.

TABLE I

NUMBER OF BINDING SITES AND DISSOCIATION CONSTANTS FOR 1-NAPHTHOYL-ATP ON TF_1, α AND β SUBUNITS

Values determined by changes of intrinsic fluorescence of TF_1 and by changes of fluorescence anisotropy of 1-naphthoyl-ATP. Binding model: sites identical and independent. Errors, given as error factors (in brackets), indicate the standard error of calculated parameters; the listed parameters are to be divided, resp. multiplied, by these error factors in order to obtain the standard variation. For details, see Figs. 3, 4 and 5, text and Materials and Methods.

Method	Number of binding sites for 1-naphthoyl-ATP			Dissociation constants K_D (1-naphthoyl-ATP)		
	TF_1	α	β	TF_1 (μM)	α (μM)	β (μM)
Anisotropy	1.6 (1.1)	1.0 (1.4)	0.7 (1.2)	0.170 (1.1)	4.6 (3.3)	1.9 (3.4)
Intrinsic fluorescence	2.0 (1.7)	–	–	0.340 (3.4)	–	–

script A symbolizes the presence of ATP). Since a 100-fold excess of ATP is necessary for complete displacement of 1-naphthoyl-ATP from the enzyme, it is concluded that ATP binds to TF_1 with a much lower affinity than 1-naphthoyl-ATP.

In contrast to TF_1, the reaction rates of 1-naphthoyl-ATP binding to isolated α and β subunits were found to be too fast to be resolved by our methods; within the mixing time of 10 s the reaction had already reached equilibrium. Therefore, only a lower limit of the rate constants could be calculated: $k_1 > 5 \cdot 10^4\ M^{-1} \cdot s^{-1}$. Similarly, the release kinetics of 1-naphthoyl-ATP in the presence of ATP were much faster than in the case of TF_1. The measured rate constants for TF_1 and the estimated values for the subunits are given in Table II.

The concentration of ATP needed for complete release of 1-naphthoyl-ATP from isolated α and β subunit, respectively, was in the same order of magnitude as the concentration of subunit-1-naphthoyl-ATP complex. This suggests similar dissociation constants (K_D) for ATP and 1-naphthoyl-ATP. This is one more striking difference between isolated α and β subunits on the one hand and TF_1 on the other.

Binding of natural ligands (ADP, ATP) to TF_1, α and β subunits

The exchangeability of bound 1-naphthoyl-ATP with excess ATP was described above. In order to determine the affinity of these sites for ADP and ATP, the corresponding experiment was performed with different concentrations of ADP and ATP (Fig. 7). In the case of TF_1, ATP obviously is more potent than ADP in displacing 1-naphthoyl-ATP from its binding sites (Fig. 7a). However, even a large excess of ATP over 1-naphthoyl-ATP (400-fold) did not result in total release of all bound 1-naphthoyl-ATP.

As stated above, in presence of natural ligands the two binding sites for 1-naphthoyl-ATP are no longer identical; therefore, the results presented in Fig. 7a could not be used to determine the dissoci-

TABLE II

RATE CONSTANTS FOR 1-NAPHTHOYL-ATP BINDING TO AND RELEASE FROM TF_1, α AND β SUBUNITS

The rate constant k_1(1-naphthoyl-ATP) for TF_1 is the average of seven measurements; k_{-1}^{A} and k_{-2}^{A} are the average of three measurements. For further details, see Fig. 6 and text.

	k_1 ($10^3\ M^{-1} \cdot s^{-1}$)	k_{-1}^{A} (s^{-1})	k_{-2}^{A} (s^{-1})
TF_1	6.6	$3 \cdot 10^{-3}$	$< 2 \cdot 10^{-4}$
α subunit	> 50	> 0.14	–
β subunit	> 50	> 0.14	–

TABLE III

DISSOCIATION CONSTANTS K_D(ATP) AND K_D(ADP) FOR α AND β SUBUNITS

Data from Fig. 7. Errors, given as error range (symmetrical error), indicate the standard error of calculated parameters. For further details see text.

	K_D(ATP) (μM)	K_D(ADP) (μM)
α subunit	8 ± 0.15	38 ± 15
β subunit	11 ± 5	7 ± 2

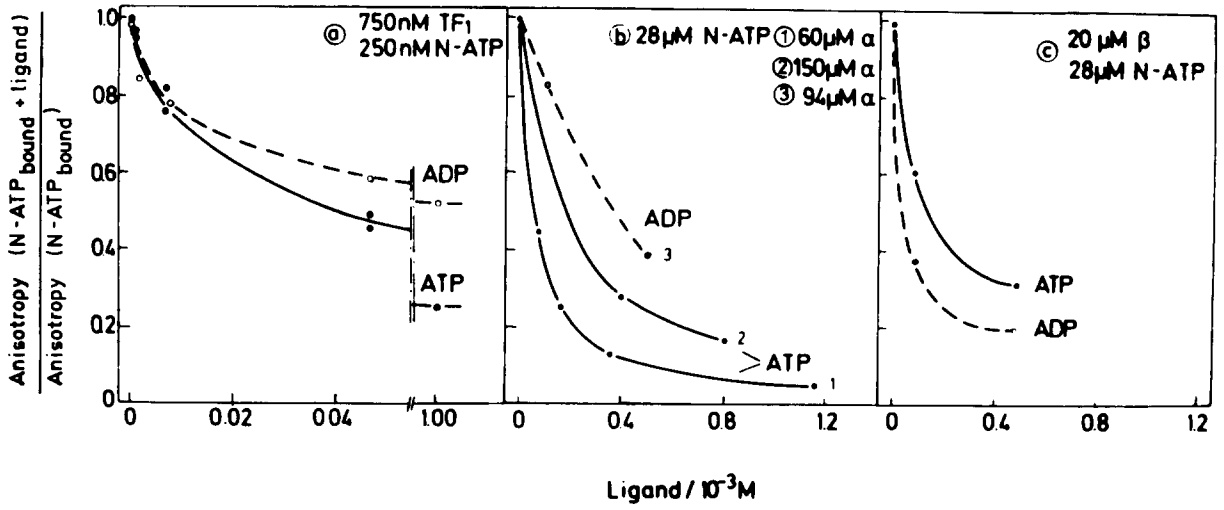


Fig. 7. Release of 1-naphthoyl-ATP, bound to TF_1, α or β subunit by addition of ATP or ADP: The anisotropy of the fluorescence of 1-naphthoyl-ATP was measured as a function of the AD(T)P-concentration after equilibrium of the reaction has been reached.

ation constants for ATP and ADP. This is different in the case of isolated α and β subunits, which were shown to have only one binding site for 1-naphthoyl-ATP (see Fig. 5). Fig. 7b and c show the ratio of 1-naphthoyl-ATP_{bound} after to 1-naphthoyl-ATP_{bound} before addition of the indicated concentrations of ADP or ATP, respectively, for isolated α and β subunits. Interestingly, ATP seems to have a higher affinity to the α subunit than ADP, while the opposite behaviour is found in the case of the β subunit. Almost all 1-naphthoyl-ATP bound to α or β can be displaced by an excess of natural ligand. Assuming a monovalent binding system, K_D(ADP) and K_D(ATP) can be calculated, as shown in Table III. While β binds ADP with a slightly higher affiity than α does, the K_D(ATP) values are nearly identical for α and β subunits.

Results shown in Table III are in harmony with data which have been obtained using circular dichroism methods [9].

Discussion

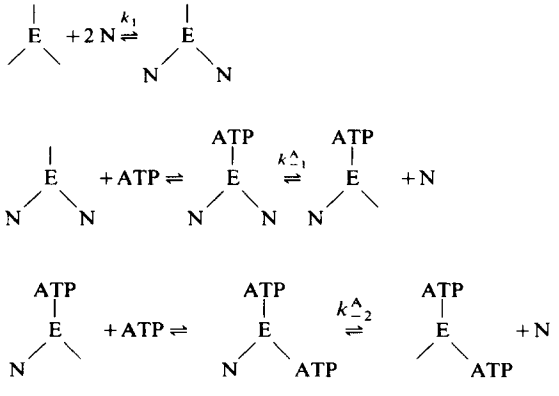
Results presented in this paper clearly indicate that nucleotide binding properties (thermodynamics and kinetics) of both α and β subunits of TF_1-ATPase are strikingly dependent upon whether these subunits are integrated in the enzyme complex or not. Interactions of α and β subunits in TF_1 result in an increase of the binding affinity for 1-naphthoyl-ATP by a factor of more than 10. As TF_1 contains 3 α and 3 β subunits [21] both of which are able to bind 1 mol 1-naphthoyl-ATP per mol subunit, one might expect six binding sites on TF_1. Actually, only two binding sites with increased binding affinities are exhibited by TF_1. Obviously, the affinity constants of the other four binding sites are reduced to such an extent that they are no longer detectable in a reasonable range of concentration. This phenomenon may be explained by at least two underlying mechanistic principles:

(1) In the ATPase complex, α and β subunits are arranged in an alternating sequence [22]. The nucleotide binding domains of an α/β pair might be orientated to each other in a way which yields only one high affinity binding site [2,27]. This would reduce the total number of binding sites which are accessible in TF_1 by a factor of two. Although the affinity of such a composite site is significantly larger as compared with an isolated subunit, the rate constant for binding 1-naphthoyl-ATP is diminished by a factor of at least 7. This may reflect the fact that a composite site on TF_1 is less freely accessible than the corresponding sites on isolated subunits. This view is in line with the finding that the fluorescence at 365 nm is increased upon binding 1-naphthoyl-ATP to TF_1, but not upon binding to the isolated subunits. As

the fluorescence increase at 365 nm is due to a second peak of the 1-naphthoyl-ATP-fluorescence at a lower wavelength, which in turn is a function of the hydrophobicity of the environment, it may be concluded that the 1-naphthoyl-ATP binding sites on TF_1 are more hydrophobic than those on the isolated subunits. Assembly of subunits in TF_1 obviously renders these sites less polar.

(2) The number of binding sites may be further reduced by anticooperative interactions between these binding sites as has been proposed also for the mitochondrial enzyme [10,23,24]. Since only two high-affinity binding sites are accessible at a given time, one has to postulate that the third site is rendered inaccessible either directly by occupation of the first two sites or indirectly via regulatory control exerted by the minor subunits. Thus, by analogy with the enzyme from bovine heart mitochondria, it is assumed that 1-naphthoyl-ATP binds to two nucleotide binding sites out of a total of three sites of high affinity which are considered to be identical a priori, i.e., in the absence of nucletides.

Additional evidence supporting these hypotheses comes from exchange experiments. Since the binding of 1-naphthoyl-ATP to the binding sites is monophasic and both sites have the same K_D, one might as well expect a monophasic release of 1-naphthoyl-ATP. In the presence of ATP, however, this is obviously not the case (Fig. 6). Hence, it may be concluded that ATP not merely binds to a site which has been left unoccupied by 1-naphthoyl-ATP, but also controls the release of 1-naphthoyl-ATP from neighbouring sites. This can be described by the following reaction sequence (E = TF_1, N = 1-naphthoyl-ATP):



It should be noted that a similar mode of displacement of 1-naphthoyl-ADP promoted by ADP via anticooperative interactions, has also been described for F_1-ATPase from bovine heart mitochondria [10,25].

Studies concerned with ligand binding to isolated subunits have been reported by various groups [9,26]. In the case of *Rhodospirillum rubrum*, one binding site for P_i and two for ADP and ATP were found on the isolated β-subunit [28,29]. Interestingly, the dissociation constants of the high affinity sites for ADP and ATP are in the same range as the dissociation constant reported here for the β subunit of TF_1. (As a restriction of the applied technique, binding sites with $K_D > 150$ μM could not be detected in our study.) Homologies in the amino acid sequence of the nucleotide binding sites which have been reported for different ATPases [30] might be the reason for similar K_D values of the isolated β subunits.

The results of this report and the hypothesis outlined above give a minimal description of the mode of nucleotide binding to TF_1. The proposed model is not the only conceivable one, but certainly a most plausible one. Its main features, reduction of the number of binding sites and alteration of their affinities by subunit–subunit interactions, agree with hypotheses of other authors [6,7,24,26].

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