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The Heterogeneous Interaction of Substoichiometric TNP-ATP and F_1-ATPase from *Escherichia coli*

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The interactions of substoichiometric TNP-ATP and F_1-ATPase from *Escherichia coli* (EF_1) were examined and compared with those in the case of mitochondrial F_1-ATPase (MF_1) and F_1-ATPase from thermophilic *Bacillus* PS3 (TF_1). EF_1 hydrolyzed substoichiometric TNP-ATP faster than TF_1 or MF_1, although some 20% of the TNP-ATP remained unhydrolyzed even in the presence of excess chase ATP. The affinity of the catalytic site of EF_1 for the product, TNP-ADP, was weaker than that of TF_1 or MF_1, and the TNP-ADP was readily released upon addition of excess ATP. The amplitude of the difference absorption spectrum induced by binding of TNP-AT(D)P to EF_1 was smaller than that of MF_1 or TF_1 under similar experimental conditions. When an excess amount of TNP-ATP was added to EF_1 and the change of the difference spectrum was measured, the shape of the difference spectrum of the ATP-replaceable fraction was very similar to that in the case of binding of TNP-ATP to the isolated β subunit of TF_1, indicating that the rapidly replaceable fraction of bound TNP-ATP was actually at the catalytic site and most of the non-replaceable portion was bound at noncatalytic sites. Weaker affinity of the catalytic site for TNP-ATP may account for the heterogeneous binding and hydrolysis under the conditions described in this paper.

Key words: *Escherichia coli*, F_1-ATPase, nucleotide binding, TNP-ATP, uni-site catalysis.

F_1-ATPase is the catalytic portion of ATP synthase, which catalyzes the synthesis of ATP from ADP and P_i in response to protonic electrochemical gradients generated by electron transport processes. F_1-ATPase is composed of five different subunits, present in a stoichiometry of $\alpha_3\beta_3\gamma\delta\varepsilon$. The total number of nucleotide binding sites on F_1-ATPase is six, of which three sites are potential catalytic sites and the other three sites are noncatalytic sites. Due to the interactions among these nucleotide binding sites, F_1-ATPase exhibits complex cooperative kinetics in ATP hydrolysis (*1-3*), as is also observed with the membrane-bound enzyme (*4, 5*) during steady-state turnover.

A prominent characteristic of this ATPase activity is observed under a substoichiometric condition where the molar amount of substrate is less than that of the enzyme. Under these conditions, Grubmeyer *et al.* reported that the ATP added substoichiometrically to mitochondrial F_1-ATPase (MF_1) bound rapidly to the enzyme and was slowly hydrolyzed. Furthermore, the hydrolysis and product release were enormously promoted by chase addition of excess ATP (chase-promotion) (*6*). This process, often called "uni-site catalysis," has been regarded as a partial reaction of the sequential alternate binding change mechanism for ATP hydrolysis postulated by Boyer (*7, 8*). But various F_1s from different sources or the use of different substrate analogues gave quite different results, even when the experiments were carried out under similar conditions. For example, F_1-ATPase from thermophilic *Bacillus* PS3 (TF_1) (*9*) and its subunit complexes, $\alpha_3\beta_3\gamma$ and $\alpha_3\beta_3\delta$ (*10*), do not exhibit promoted hydrolysis of ATP by chase-addition of excess ATP at room temperature and little, if any, at 60°C. On the other hand, when TNP-ATP was used as a substrate, TF_1 exhibited clear chase-promotion of hydrolysis, but the product, TNP-ADP, hardly dissociated from the enzyme (*11*). In the case of MF_1, clear chase-promotion was observed with both ATP (*6*) and TNP-ATP (*12*). However, when TNP-ATP was used as a substrate, the hydrolyzed product, TNP-ADP, hardly dissociated from the enzyme as in the case of TF_1. Based on an analysis of the relationship between the occupancy of the catalytic sites by TNP-AT(D)P and the inhibition of the steady-state ATPase activity of MF_1, we have proposed that the multiple catalytic sites mutually activate each other when they are occupied by substrates (*12*). The F_1-ATPase from *Escherichia coli* (EF_1) hydrolyzed substoichiometric ATP faster than other F_1s and the extent of chase-promotion was relatively small (*13-15*). By comparing the experimental data and computer simulation, we identified heterogeneity of the hydrolysis of substoichiometric ATP by EF_1 (*15*). Recently, Xiao and Penefsky indicated that only the fraction of EF_1 which is devoid of δ subunit exhibits chase-promotion and a

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Abbreviations: F_1, a water-soluble part of H^+-ATP synthase; EF_1, F_1 from *Escherichia coli*; TF_1, F_1 from thermophilic *Bacillus* PS3; MF_1, F_1 from beef heart mitochondria; TNP-ATP and TNP-ADP, the 2′,3′-*O*-(2,4,6-trinitrophenyl) derivatives of ATP and ADP; ΔA, the size of difference absorbance change at the indicated wavelength.

mixture of δ-containing and δ-less EF_1 actually results in heterogeneous hydrolysis of substoichiometric ATP (*16*). Thus, the differences in the profile of "uni-site" and "multi-site" hydrolysis of ATP or TNP-ATP observed under various conditions might be caused by the different nature of F_1s (*e.g.* EF_1, TF_1, and MF_1) or by the different nature of the substrates.

In the present study, we have examined the hydrolysis of substoichiometric TNP-ATP by δ-containing EF_1 for comparative purposes to determine whether it gives different results from other F_1s or not. The results indicated that EF_1 hydrolyzed TNP-ATP in a quite similar way to ATP. As is the case for ATP, the hydrolysis of substoichiometric TNP-ATP by EF_1 proceeded faster than that by TF_1 or MF_1, and some 20% of the TNP-ATP remained unhydrolyzed. The hydrolyzed product, TNP-ADP, bound rather weakly to EF_1, as it readily dissociated upon addition of excess ATP. Calculation of the difference spectra of the ATP-replaceable fraction of bound TNP-ATP revealed that the shape of the spectrum was strikingly similar to that raised by binding of TNP-ATP to the isolated β subunit of TF_1. The data are discussed in comparison with those for other F_1s.

MATERIALS AND METHODS

Preparation of EF_1—EF_1 was prepared according to Wise (*17*) with some modifications as described by Kato *et al.* (*18*). The subunit composition of EF_1 preparation was analyzed by SDS-PAGE and consisted of apparently 5 kinds of subunits. Specific activity at 25°C in the presence of 5 mM ATP was about 30 U/mg. Before use, the enzyme was desalted on a Sephadex G-25 fine column (1.5×20 cm) equilibrated with 20 mM Tricine-KOH, pH 8.0. Endogenous nucleotides were quantitated by reversed-phase HPLC after extraction of nucleotides from the protein with perchloric acid as described below. The EF_1 contained about 1 mol of adenine nucleotide per mol.

Preparation of TNP-ATP—TNP-ATP was synthesized as described (*19*) and purified (*20*). The purity of the analog was checked by HPLC (described below).

Measurement of Difference Spectra—Difference spectra induced by the interaction between TNP-ATP and the enzymes were measured using a double-beam spectrophotometer, model UV-2200 (Shimadzu, Kyoto) as described (*11, 12, 21*).

Analysis of TNP-ATP, TNP-ADP, and Adenine Nucleotides by HPLC—The amounts of TNP-ATP, TNP-ADP, and adenine nucleotides were determined by using reversed-phase HPLC on a TSK ODS-80$_{TM}$, (Tosoh, Tokyo) or a Cosmosil 5C_{18}-AR (Nacalai Tesque, Kyoto) as described (*11*).

TNP-ATP Hydrolysis under Substoichiometric Conditions—The experiments were carried out at 25°C. The reaction was initiated by injecting 50 μl of desalted EF_1 solution (1 μM) into 50 μl of vigorously stirred reaction mixture which contained 0.25 μM TNP-ATP, 20 mM Tricine-KOH, and 2 mM $MgCl_2$, pH 8.0. The reaction was quenched by the addition of 7.5 μl of 24% perchloric acid (acid quench). In chase experiments, 50 μl of 30 mM Mg-ATP solution dissolved in the same buffer as TNP-ATP was added to the reaction mixture at the indicated time. After 5 s, the reaction was quenched by the addition of 7.5 μl of 24% perchloric acid (ATP-chase). The quenched solutions were placed on ice and the precipitated proteins were removed by centrifugation. A portion of the supernatant was injected directly onto the HPLC column for analysis of TNP-adenine nucleotides. TNP-ATP was stable without neutralization for at least 2 days when stored at 4°C.

RESULTS AND DISCUSSION

Hydrolysis of Substoichiometric and Stoichiometric TNP-ATP by EF_1—Comparison with TF_1 and MF_1—As shown in Fig. 1, A and B, the hydrolysis of substoichiometric TNP-ATP by EF_1 was not monophasic and there was a residual fraction, of which hydrolysis was not promoted by ATP-chase. The same result was obtained when the molar ratio of TNP-ATP to EF_1 was decreased from 1 : 4 to 1 : 10 (data not shown). As a rough estimation, the time course of the acid quench experiment was analyzed as a sum of three components. About 50% of the TNP-ATP was hydrolyzed quickly with an apparent first-order rate constant of 4×10^{-1} s^{-1}, whereas 30% of the total was hydrolyzed 30 times more slowly, with a rate constant of 1.3×10^{-2} s^{-1}, and 20% remained unhydrolyzed (Fig. 1, A and B). Simulation with two exponential components or one exponential and one constant did not give a satisfactory fit. The 20%-fraction seems to correspond to TNP-ATP which remained unhydrolyzed in the presence of chase-ATP (Fig. 1A). The time courses of the hydrolysis of substoichiometric TNP-ATP by TF_1 and MF_1 are also shown in Fig. 1B for reference. The unhydrolyzed fraction was also observed in the case of TF_1 (Fig. 1B and Ref. *11*). Some portion of TNP-ATP, which was added under a substoichiometric condition, might have bound to noncatalytic sites also in the case of TF_1. On the other hand, the hydrolysis reached nearly completion after 300 s in the case of MF_1 (Fig. 1B and Ref. *12*).

The apparent rate constant of TNP-ATP hydrolysis of the main hydrolyzable fraction by EF_1 (4×10^{-1} s^{-1}) was much bigger than the corresponding rate constant of MF_1 (1.4×10^{-2} s^{-1}, Ref. *12*) or TF_1 (1.5×10^{-2} s^{-1}, calculated from the data in Ref. *11*). When substoichiometric ATP was used as the substrate, the majority of the substrate was also rapidly hydrolyzed (*13-15*) by EF_1. These results indicate that the heterogeneous binding of TNP-ATP occurs on EF_1 even under substoichiometric conditions, as is the case for ATP. Thus, the differences between the hydrolysis of substoichiometric substrates by EF_1 (*13-15*) and by TF_1 (*11*) or MF_1 (*12*) reflects mainly the difference between EF_1 and other F_1s, rather than the difference of the substrates used, ATP and TNP-ATP. The presence of a rapidly hydrolyzed fraction and the small extent of chase-promotion observed for both ATP and TNP-ATP can be explained by the relative difference of affinity between the catalytic sites and the noncatalytic sites. Probably, the affinity of the first catalytic site is not much higher than that of the other catalytic sites or noncatalytic sites and substoichiometric TNP-ATP is distributed among these sites. It has been reported that the affinity of the first catalytic site of EF_1 for ATP (*22*) was two orders weaker than that of MF_1 (*6*). As for the isolated subunits of EF_1, it was reported that the α subunit has a lower K_d for ATP (10^{-7} M, Ref. *23*) than the β subunit (10^{-5} M, Ref. *24*).

The effect of P_i on the hydrolysis of substoichiometric substrates seems to depend on the conditions. In the case of TF_1, P_i does not affect the hydrolysis of substoichiometric TNP-ATP (Hisabori, T., unpublished results) or ATP (*9*), whereas P_i is required for the chase-promotion in the case of MF_1 when ATP is used as the substoichiometric substrate (*6*) and not when TNP-ATP is used (*15*). On the other hand, we reported that P_i diminished the rate of binding of substoichiometric ATP and inhibited the hydrolysis of 1 μM ATP by a catalytic concentration of EF_1 (*15*). When TNP-ATP was used as a substrate for EF_1, hydrolysis of the substoichiometric substrate and chase-promotion were slightly enhanced by the addition of 5 mM P_i (data not shown).

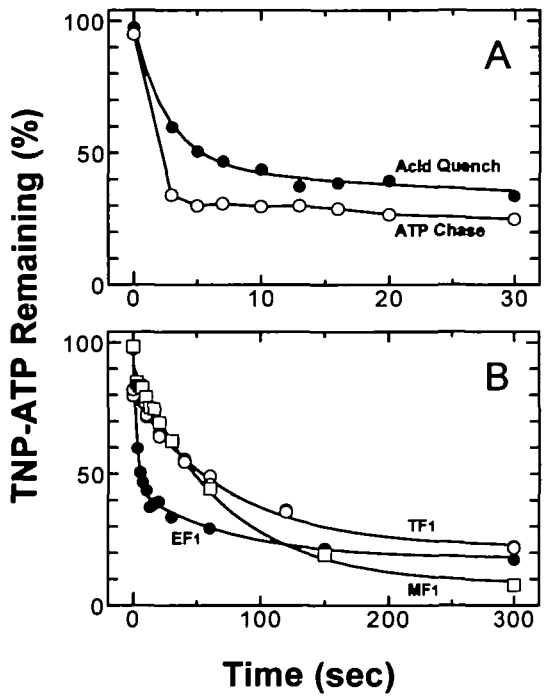

Fig. 1. **Hydrolysis of substoichiometric TNP-ATP by EF_1, TF_1, and MF_1.** A: Hydrolysis of substoichiometric TNP-ATP by EF_1 and the effect of ATP-chase. Acid quench and ATP chase experiments were carried out as described in "MATERIALS AND METHODS." In acid quench experiments (●), the reaction was quenched by the addition of perchloric acid at the indicated times. In chase experiments (○), Mg-ATP solution was added to the reaction mixture at the indicated times. After 5 s, the reaction was quenched by the addition of perchloric acid. Time zero corresponds to the experiment in which a mixture of TNP-ATP and perchloric acid or a mixture of TNP-ATP and chase ATP was added to the EF_1 solution for the acid quench and ATP-chase experiments, respectively. The line for acid quench is the simulated curve according to the parameters determined from the data in B (●). In order to indicate clearly the effect of ATP chase, only the time course up to 30 s is shown. B: Comparison of the hydrolysis of substoichiometric TNP-ATP by EF_1, TF_1, and MF_1. The time courses of acid quench up to 300 s were analyzed as a sum of exponentials and a constant. The solid lines are theoretically calculated ones. EF_1 (●), 53% of the fraction decayed with an apparent first-order rate constant of 0.37 s^{-1}, 27% with a rate constant of 0.013 s^{-1}, and 17% remained constant. The experimental data are the same as for A. TF_1 (○), 58% decayed with a rate constant of 0.013 s^{-1} (average of independent experimental results was 0.015 s^{-1} as described in the text) and 22% remained constant. MF_1 (□), 83% decayed with a rate constant of 0.014 s^{-1} and 7.9% remained constant. Since the experimental values at 0 s were not exactly 100%, the sums of the theoretically deduced components are less than 100%.

The extent of hydrolysis of TNP-ATP was examined as a function of TNP-ATP concentration from the substoichiometric range to a molar excess condition (Fig. 2). The extent of hydrolysis in 5 s was almost the same (about 30%) when the molar ratio of TNP-ATP to EF_1 was below 2 and then it decreased at higher molar ratio. This is in contrast to the results obtained for MF_1 and $\alpha_3\beta_3\gamma$ complex of TF_1 (Muneyuki, E., Honda, M., and Yoshida, M., unpublished results), which exhibited increases in the extent of hydrolysis with increase of total TNP-ATP concentration. Since the ordinate in Fig. 2 stands for the ratio of hydrolyzed TNP-ATP to the total amount of TNP-ATP added, the increase of % TNP-ATP hydrolyzed with increase of TNP-ATP concentration is observable only when there is strong positive catalytic cooperativity. We suspect that the apparent absence of positive catalytic cooperativity for EF_1 in this experiment is caused by the relatively rapid hydrolysis and rapid release in the absence of promoter-nucleotide and the lower affinity of the catalytic sites for TNP-adenine nucleotides as observed in Fig. 1. That EF_1 has lower affinity for TNP-ATP than other F_1s is consistent with the difference spectrum measurements (see below).

Difference Spectra Induced by Binding of TNP-ATP to EF_1—As reported previously, the difference spectra induced by binding of TNP-ATP to the isolated α subunit of TF_1 has a higher peak at 514 nm, a trough at about 450 nm and a lower peak at 410 nm, and that to the β subunit has a lower peak at about 510 nm, a higher peak at 420 nm and a deep trough at 395 nm (*11*). The difference spectra induced by binding of substoichiometric TNP-AT(D)P[2] to EF_1 has a prominent peak at around 520 nm and a shallow trough at around 450 nm, a lower peak at around 420 nm and a deep trough at around 400 nm (Fig. 3A). The higher peak at 520 nm is similar to that induced by binding to the isolated TF_1 α subunit and the deep trough at around 400

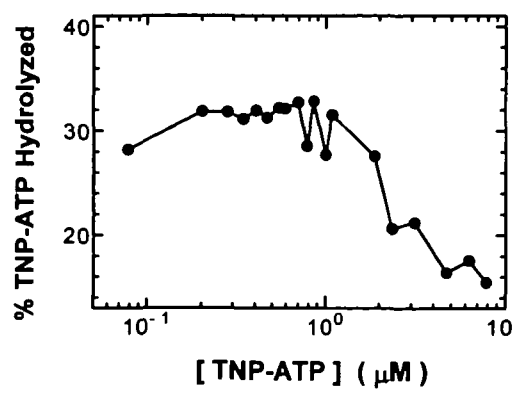

Fig. 2. **Hydrolysis of TNP-ATP in the concentration range between 0.075 and 7.5 μM by 0.5 μM EF_1.** Aliquots of 50 μl of EF_1 solutions (1 μM) dissolved in 20 mM Tricine-KOH, 5 mM NaP_i (pH 8.0), were mixed with 50 μl of TNP-ATP solutions (0.15 to 15 μM) in 20 mM Tricine-KOH, 2 mM $MgCl_2$, 5 mM NaP_i (pH 8.0), with vigorous stirring. After 5 s, 7.5 μl of 24% perchloric acid was added to quench the reaction and the resultant TNP-ATP and TNP-ADP were analyzed by reversed-phase HPLC.

[2] Under the experimental condition, most of the TNP-ATP added was hydrolyzed to TNP-ADP.

nm is similar to that induced by binding of TNP-ATP to the TF_1 β subunit, suggesting that TNP-AT(D)P bound to both α and β subunits under the experimental condition. In view of the heterogeneous hydrolysis of substoichiometric TNP-ATP as shown in the previous section, it is likely that TNP-ATP is distributed between catalytic (β subunits) and noncatalytic (α subunits) sites under the substoichiometric condition and the unhydrolyzed fraction (20%) seems to correspond to the TNP-ATP bound at a noncatalytic site.

The difference spectra significantly diminished upon addition of excess ATP (Fig. 3A, lower traces). This result indicates that most of the hydrolyzed product, TNP-ADP, dissociates from the enzyme. The time course of binding and dissociation was followed by examining the difference in the absorbance at 420 and at 395 nm (Fig. 3B). The binding of TNP-ATP reached equilibrium within 5 s and replacement by chase-ATP occurred within 30 s. These properties are in contrast with those with TF_1 and MF_1, in which TNP-ATP bound at the first high-affinity site hardly dissociates after addition of excess ATP (*11, 12*). This again indicates that the affinity of the site for TNP-AT(D)-P relative to ATP is lower in the case of EF_1 than TF_1 or MF_1.

Furthermore, the amplitude of the difference spectra induced by binding of substoichiometric TNP-AT(D)P to EF_1 was smaller than that obtained with TF_1 or MF_1 under similar conditions. The difference of the magnitude of the spectra was evident when the difference spectra were measured as a function of TNP-ATP concentration (Fig. 4A). In the case of TF_1 and MF_1, the amplitude of the difference spectra ($\Delta A_{425}-\Delta A_{395}$) was 0.007 to 0.008 when 2 μM enzyme was mixed with 2 μM TNP-ATP (light path=5 mm), whereas the combination of EF_1 and TNP-ATP generated a spectrum with the amplitude of only 0.004 (Fig. 4A). As catalytic and noncatalytic sites might have different affinities for nucleotides and these sites show characteristic difference absorption spectra when TNP-ATP binds to them, we expected a change of the shape of the difference spectra with increasing concentrations of TNP-ATP. We calculated the difference of the spectra between each addition of TNP-ATP to find the transition of their shape (data not shown); however, the shape of the spectrum remained essentially the same when TNP-ATP was added in a stepwise manner. As stated above, the amplitude of the difference spectra was smaller than that of TF_1 or MF_1 and there was no clear indication of saturation until the molar ratio of TNP-ATP to EF_1 exceeded 4. Furthermore, the generation of the difference spectra was significantly suppressed in the presence of 50 μM ATP (data not shown). These results also suggest that the affinity of the catalytic sites for TNP-adenine nucleotides is lower than that of TF_1 or MF_1. When 0.5 mM ATP was added to EF_1 solution (2 μM) containing 10 μM TNP-ATP, $\Delta A_{425}-\Delta A_{395}$ almost vanished (Fig. 4A). However, the difference spectrum did not become flat; indeed, the shape of the spectrum changed significantly at this point (Fig. 4B, a and b). In order to determine the difference spectrum generated by TNP-adenine nucleotide which was displaced upon addition of excess ATP, we calculated the difference of the spectra before and after the addition of ATP (Fig. 4B, c). The shape of the difference spectrum of the ATP-replaceable fraction was strikingly similar to that induced by binding of TNP-ATP to isolated β subunit of TF_1 (Fig. 2B in Ref. *11*). On the other hand, the difference spectrum after ATP addition seems to be mostly due to the TNP-AT(D)P bound to the α (noncatalytic sites) subunit, although there might have been some portion of TNP-AT(D)P bound to β subunits judging from the small trough at around 395 nm. This observation strongly suggests that the original spectrum was a combination of two spectra generated by binding of TNP-adenine nucleotide to α and β subunits. Although a direct comparison may be difficult, it seems likely that the unhydrolyzed fraction (some 20% of the total) and hydrolyzed fraction (with an apparent rate of 1.3×10^{-2} s^{-1} and with an apparent rate of 4×10^{-1} s^{-1}) observed in the hydrolysis of the substoichiometric TNP-ATP (Fig. 1) correspond to the heterogeneous binding sites revealed in the measurement of difference spectra (Fig. 4,

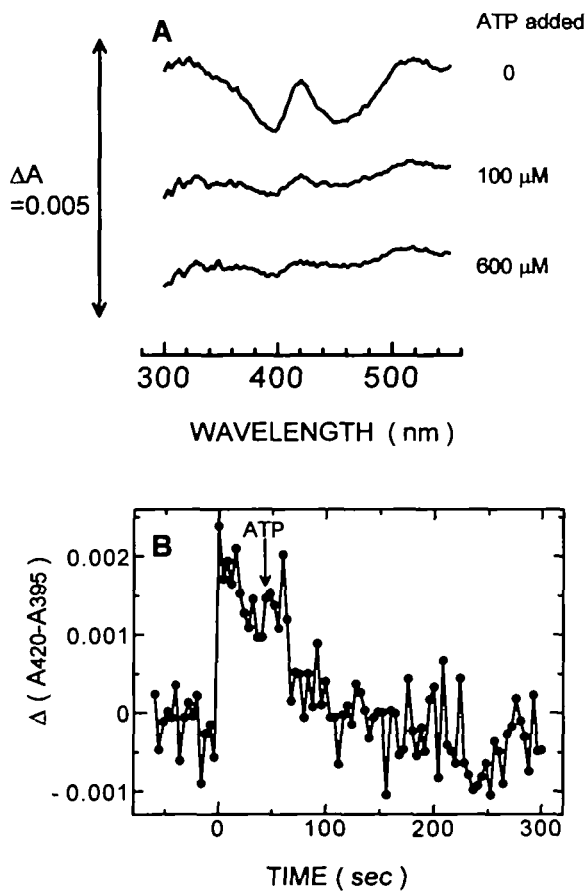

Fig. 3. **A: Difference spectra of TNP-ATP induced by binding to EF_1 under substoichiometric conditions.** Concentrated TNP-ATP solution was added to EF_1 (2 μM) in 20 mM Tricine-KOH and 2 mM $MgCl_2$, pH 8.0, to a final concentration of 0.5 μM. After 5 to 10 min, difference spectra were measured three times sequentially and averaged. The effect of ATP on the difference spectra was examined by adding small aliquots of Mg-ATP solutions. **B: Time course of binding of TNP-ATP and release induced by addition of ATP measured in terms of absorption change.** EF_1 solution (1 μM) in 20 mM Tricine-KOH and 2 mM $MgCl_2$, pH 8.0, was continuously stirred with a magnetic stirrer and the absorbances at 420 and 395 nm were measured alternately at intervals of 4 s. Then, a concentrated TNP-ATP solution was added to a final concentration of 0.5 μM at 0 time, and after 40 s, ATP was added (final 100 μM) as indicated by the downward arrow. The magnitude of ($A_{420}-A_{395}$) was plotted against time.

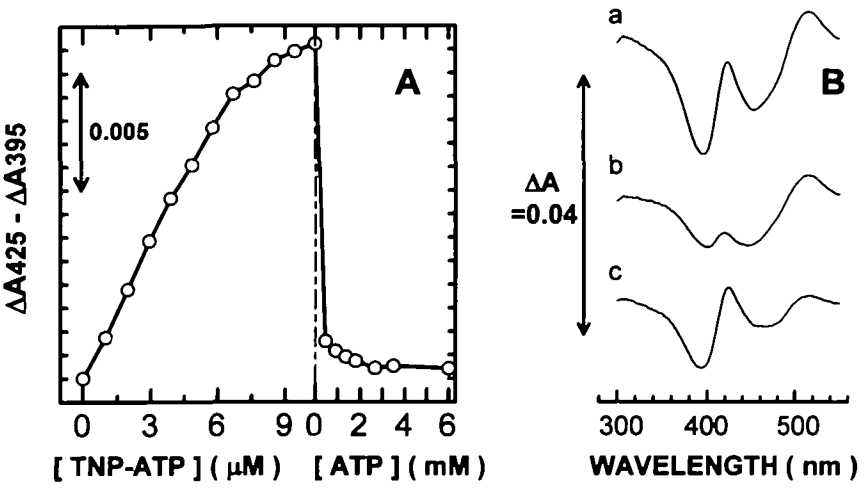

Fig. 4. **A: Difference spectra induced by binding of TNP-ATP to EF_1.** Small aliquots of concentrated TNP-ATP solution were added successively to EF_1 solution (2 μM) and after 4 min, difference spectra were measured. ΔA_{425} and ΔA_{395} were calculated and their difference was plotted. Small aliquots of TNP-ATP solutions were added successively to EF_1 solution (left side) and then concentrated Mg-ATP solutions were added. **B: Calculated difference spectrum induced by ATP-replaceable portion of TNP-ATP.** The difference spectra are those of 2 μM EF_1 plus 10 μM TNP-ATP (a), 2 μM EF_1 plus 10 μM TNP-ATP and 0.5 mM ATP (b), and subtraction of b from a which corresponds to the difference spectrum induced by the ATP-replaceable fraction of TNP-ATP (c).

ATP-non-replaceable binding sites mainly on the α subunits, and ATP-replaceable binding sites on β subunits).

As stated in the introduction, combinations of F_1s from various sources with ATP or TNP-ATP gave quite different results in the so-called "uni-site" experiments. For example, when EF_1 hydrolyzed substoichiometric ATP, the majority of the substrate was rapidly hydrolyzed and there was poor promotion of the hydrolysis by the chase-addition of ATP, whereas when TF_1 or MF_1 hydrolyzed substoichiometric TNP-ATP, the hydrolysis proceeded relatively slowly which was greatly accelerated by chase-addition of excess ATP. In the present study, it has become clear that the difference arises mainly from the difference between EF_1 and other F_1s and does not depend so much on the difference between TNP-ATP and ATP. EF_1 and other F_1s seem to be different in the relative affinity of the catalytic and noncatalytic sites for the substoichiometric substrate. In TF_1 and MF_1, the first catalytic site has by far the highest affinity for TNP-ATP and substoichiometric TNP-ATP mainly binds to the first catalytic site. The first catalytic site can retain the hydrolyzed product, TNP-ADP, even in the presence of excess ATP and the situation makes the pattern of difference spectra and kinetics of hydrolysis rather simple. On the other hand, in the case of EF_1, it seems that the affinity of the first catalytic site is not much higher than that of the second catalytic site or noncatalytic sites, resulting in heterogeneous binding and hydrolysis of the substoichiometric substrate.

In the present study, we have mainly focused on the interaction of EF_1 with TNP-ATP under a substoichiometric condition and found that it is quite different from that of MF_1 and TF_1. Recently, Weber and Senior reported the interaction of a catalytic concentration of EF_1 with excess TNP-ATP (*25*). Although direct comparison of our results with theirs may be difficult, they also found a significant difference between the interaction of EF_1 with TNP-ATP and the interaction of MF_1 with TNP-ATP reported by Grubmeyer and Penefsky (*20, 26*).

As the change of the affinity for adenine nucleotide is supposed to be an essential part of energy transduction during ATP synthesis (*8*), it is of interest to know how the difference of affinity of each adenine nucleotide binding site in EF_1 and the difference from other F_1s arise in terms of molecular structure (*27*).

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