Biochimica et Biophysica Acta, 1058 (1991) 304–311

ADONIS 0005272891001642

BBABIO 43424

Heterogeneous hydrolysis of substoichiometric ATP by the F_1-ATPase from *Escherichia coli*

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(Received 17 January 1991)

Key words: ATP hydrolysis; ATPase, F_1-; Phosphate, inorganic; ATP, substoichiometric; (*E. coli*)

The hydrolysis of 0.3 μM [α,γ-^{32}P]ATP by 1 μM F_1-ATPase isolated from the plasma membranes of *Escherichia coli* has been examined in the presence and absence of inorganic phosphate. The rate of binding of substoichiometric substrate to the ATPase is attenuated by 2 mM phosphate and further attenuated by 50 mM phosphate. Under all conditions examined, only 10–20% of the [α,γ-^{32}P]ATP that bound to the enzyme was hydrolyzed sufficiently slowly to be examined in cold chase experiments with physiological concentrations of non-radioactive ATP. These features differ from those observed with the mitochondrial F_1-ATPase. The amount of bound substrate in equilibrium with bound products observed in the slow phase which was subject to promoted hydrolysis by excess ATP was not affected by the presence of phosphate. Comparison of the fluxes of enzyme-bound species detected experimentally in the presence of 2 mM phosphate with those predicted by computer simulation of published rate constants determined for uni-site catalysis (Al-Shawi, M.D., Parsonage, D. and Senior, A.E. (1989) J. Biol. Chem. 264, 15376–15383) showed that hydrolysis of substoichiometric ATP observed experimentally was clearly biphasic. Less than 20% of the substoichiometric ATP added to the enzyme was hydrolyzed according to the published rate constants which were calculated from the slow phase of product release in the presence of 1 mM phosphate. The majority of the substoichiometric ATP added to the enzyme was hydrolyzed with product release that was too rapid to be detected by the methods employed in this study, indicating again that the F_1-ATPase from *E. coli* and bovine heart mitochondria hydrolyze substoichiometric ATP differently.

1. Introduction

The proton-translocating F_0F_1-ATP synthases of energy-transducing membranes catalyze the synthesis of ATP in response to proton electrochemical gradients generated by electron transport processes. The enzymes are comprised of an integral membrane portion, F_0, which mediates proton translocation, and a peripheral component, F_1, on which the catalytic sites reside. In its soluble form, which is more amenable to detailed kinetic analysis, F_1 catalyzes net ATP hydrolysis. The F_1-ATPases are comprised of five different polypeptide chains, designated α–ϵ in order of decreasing M_r. The subunit stoichiometry is $\alpha_3\beta_3\gamma\delta\epsilon$. In enzymes isolated from different species, the α, β and γ subunits are each products of common evolving genes, whereas the δ and ϵ subunits are not [1]. The molecular weight of the F_1-ATPase isolated from the plasma membranes of *E. coli* (EF_1), based on the stoichiometry and amino acid sequences of its constituent subunit is 380000 [1,2].

Extensive studies on the isolated F_1-ATPase have been made to elucidate the molecular mechanism of the synthase on the assumption that the hydrolytic reaction catalyzed by soluble F_1 is the reverse of ATP synthesis. By adding ATP to MF_1 under substoichiometric conditions, which allowed less than one catalytic site to be occupied, the following characteristic features were revealed by Penefsky and his colleagues [3–5]. (1) ATP binds to a single catalytic site rapidly with very high affinity. (2) The equilibrium constant for the interconversion of enzyme-bound products at the high-affinity catalytic site is close to unity. (3) In the absence of promotion caused by adding excess ATP to bind to a second catalytic site, product release from the

The computer program used in the simulation is deposited in the BBA Data Deposition under No. BBA/DD/435/43424 (1991) 1058/304. Kindly apply to the BBA Editorial Secretariat, P.O. Box 1345, 1000 BH Amsterdam, The Netherlands.

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single site is extremely slow. (4) Hydrolysis of ATP bound to the single site and release of the products are enormously enhanced when a second catalytic site is occupied by the addition of excess ATP. This process, which is known as uni-site catalysis, is considered to be a partial reaction of the binding change mechanism for ATP hydrolysis postulated by Boyer and his colleagues [6,7]. The binding change mechanism was postulated to provide an explanation for the disappearance of the intermediate $P_i \rightleftharpoons HOH$ oxygen exchange reaction as the ATP concentration is increased from 1 to 100 μM. Whether cooperative ATP hydrolysis proceeds with three equivalent catalytic sites participating sequentially, as postulated by the Boyer's laboratory or with two of the potential catalytic sites hydrolyzing ATP cooperatively and the third playing a regulatory role, as suggested independently by Van Dongen and Berden [8] and Fromme and Gräber [9], remains unsettled.

The basic characteristics of hydrolysis of substoichiometric ATP displayed by MF_1, which according to Penefsky and Grubmeyer [5] are observed only after activation with millimolar concentrations of P_i, are not exhibited by all F_1-ATPases. When substoichiometric [α,γ-^{32}P]ATP is hydrolyzed by the F_1-ATPase isolated from the plasma membranes of the thermophilic bacterium PS3 (TF_1) at 23 ° C in the presence or absence of P_i, substrate binding is rate-limiting [10]. Consequently, bound, radioactive products are not detected. Furthermore, promotion of catalysis by excess non-radioactive ATP is not observed. In experiments with TF_1 at 60 ° C, addition of excess non-radioactive ATP to enzyme loaded with substoichiometric radioactive ATP caused about 10% promotion of hydrolysis of radioactive substrate. However, tightly bound products were not detected at this temperature after passing reaction mixtures through centrifuge columns of Sephadex. Therefore, the characteristics of hydrolysis of substoichiometric ATP catalyzed by TF_1 and MF_1 differ considerably. On the other hand, Kasho et al. [11] have shown that TF_1 exhibits intermediate $P_i \rightleftarrows$ HOH exchange during ATP hydrolysis that increases significantly as the concentration of substrate is decreased. These results suggest that the uni-site catalysis may not necessarily be related to the oxygen exchange reactions. Hydrolysis of substoichiometric ATP by the F_1-ATPase isolated from the plasma membranes of *E. coli* (EF_1) have been examined in the laboratories of Senior [12–14] and Futai [15,16]. Although in these laboratories it was observed that hydrolysis of substoichiometric ATP by EF_1 is heterogeneous, these workers concluded that it proceeds in essentially the same way as described for MF_1 [12–16]. However, the amount of enzyme-bound ^{32}P species detected in these studies were much less than those observed in studies with MF_1 under comparable conditions [3–5].

This report compares hydrolysis of substoichiometric ATP by EF_1 in the presence and absence of P_i using [α,γ-^{32}P]ATP as substrate in order to follow the fate of both products. The experimental results obtained in the presence of P_i are compared with computer simulations based on rate constants reported by Al-Shawi et al. [13,14]. Although the experimental results in this study are in good agreement with those reported from the Senior [12–14] and Futai [15,16] laboratories, the computer simulations of the rate constants reported for hydrolysis of substoichiometric ATP by EF_1 [13,14] are inconsistent with the experimental results reported here and by others [12–16]. These discrepancies indicate that the majority of substoichiometric ATP added to EF_1 is hydrolyzed by a process other than from which the rate constants were obtained.

Experimental procedures

Materials. The [α-^{32}P]ATP and [γ-^{32}P]ATP were purchased from New England Nuclear or Amersham International. Other chemicals were of the highest grade commercially available. EF_1 was the generous gift of Drs. Massimo Tommasino and Roderick Capaldi. It hydrolyzed 14 μmol of ATP per mg per min when assayed at 30 ° C. When submitted to polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate and stained with Coomassie brilliant blue, the enzyme was found qualitatively to be deficient in, but not depleted of, the ϵ subunit. After passing the enzyme through a centrifuge column of Sephadex G-50 equilibrated with Tris-H_2SO_4 (pH 7.5)/1 mM EDTA, the endogenous nucleotide content was determined to be 1.8 mol of ADP and 2.6 mol of ATP per mol of EF_1.

Preparation and treatment of reaction mixtures for hydrolysis of substoichiometric [α,γ-^{32}P]ATP. Before use in experiments, EF_1 was passed through a 1.0 ml centrifuge column of Sephadex G-25 equilibrated with 50 mM Hepes-NaOH buffer (pH 7.4)/0.5 mM $MgSO_4$. The gel-filtered enzyme solution was diluted to contain 1.1 μM EF_1, in 50 mM Hepes-NaOH (pH 7.4)/0.5 mM $MgSO_4$, with or without 2 mM sodium phosphate. In these experiments, 44 μl of 1.1 μM EF_1 was pipetted directly into 6 μl of 2.5 μM [α,γ-^{32}P]ATP (0.3 μM final concentration), prepared as described [17] in a 10 × 75 mm test-tube using a Gilson Pipetman. For each of the points indicated in the figures, a separate reaction mixture was prepared and incubated at 23 ° C. In acid-quench experiments, reactions were terminated by adding 10 μl of 14% perchloric acid directly, whereas in cold chase experiments they were terminated by adding 10 μl of 14% perchloric acid 10 s after addition of 5.5 μl of 50 mM MgATP. To determine bound species, reaction mixtures were passed through 5 ml centrifuge columns, equilibrated with 50 mM Hepes-NaOH (pH 7.4)/0.5 mM $MgSO_4$, directly into 20 μl of

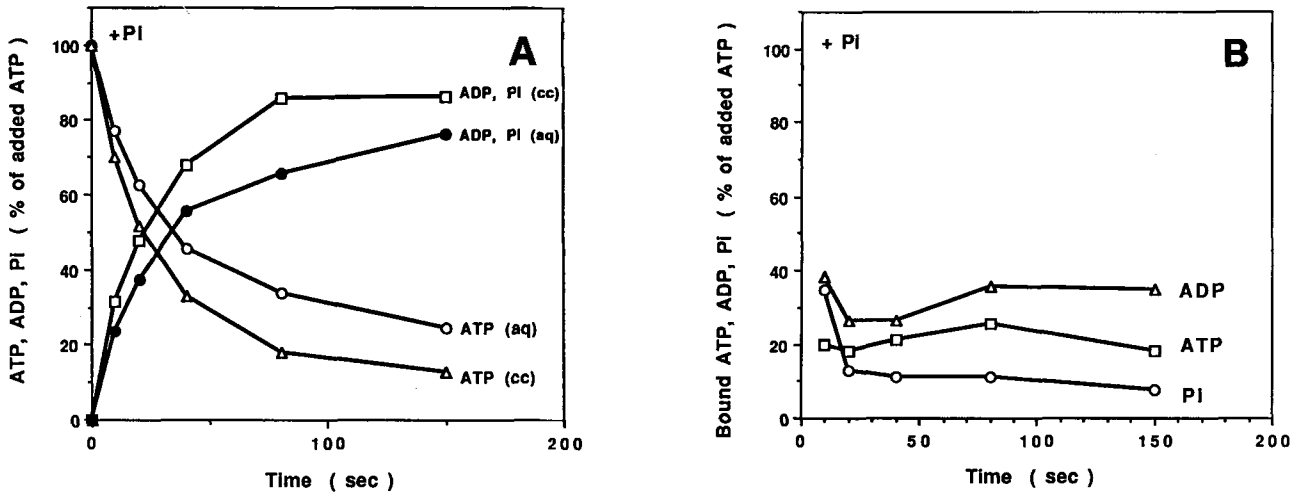

Fig. 1. Hydrolysis of substoichiometric [α,γ-^{32}P]ATP by EF_1 in the presence of 2 mM P_i. The reaction mixtures were prepared as described under Experimental procedures. (A) Fluxes of bound plus free radioactive species. The abbreviations on the figure are: (cc), cold chase; and (aq), acid quench. The ADP, P_i curves represent the averages of the values for ADP and P_i obtained in the acid quench and cold chase experiments. (B) Fluxes of bound radioactive species.

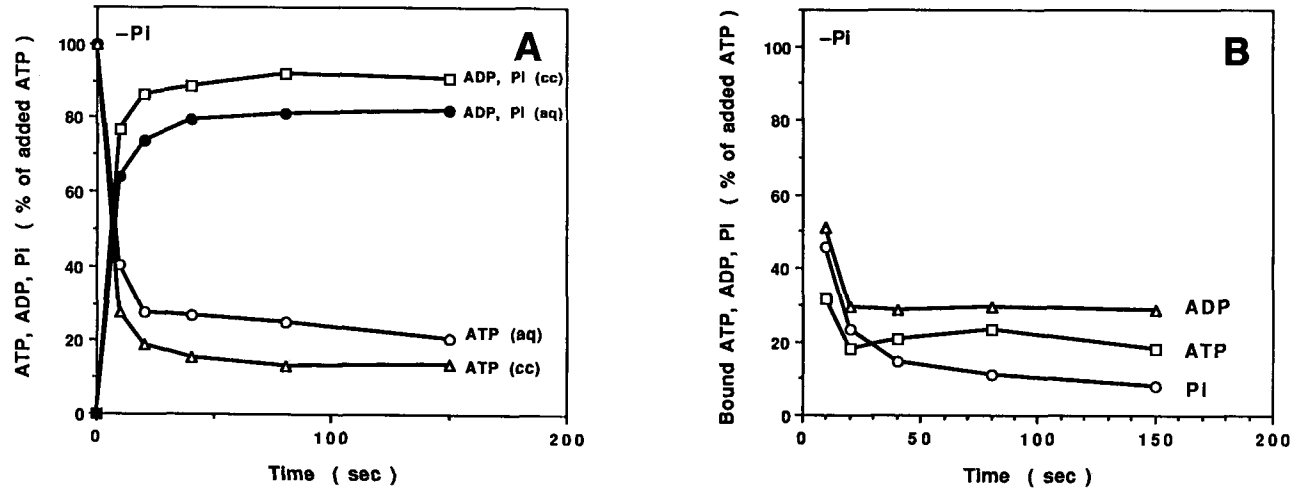

Fig. 2. Hydrolysis of substoichiometric [α,γ-^{32}P]ATP by EF_1 in the absence of P_i. The reaction mixtures were prepared as described under Experimental procedures. The abbreviations used are the same as those of Fig. 1. (A) Fluxes of bound plus free radioactive species. (B) Fluxes of bound radioactive species.

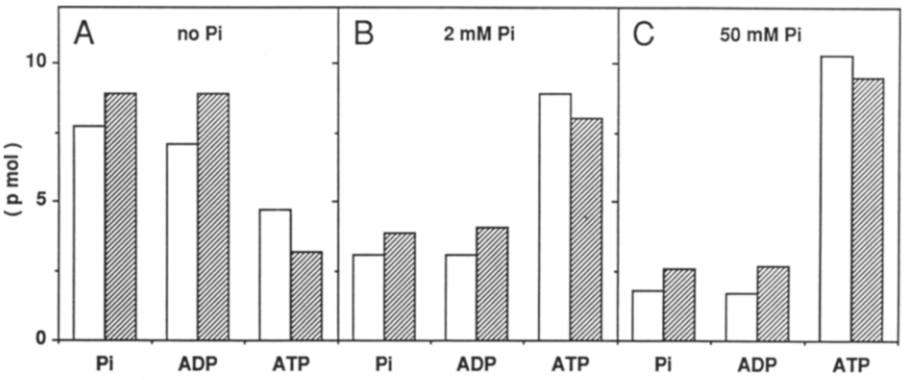

Fig. 3. The effect of 2 mM and 50 mM P_i on hydrolysis of substoichiometric [α,γ-^{32}P]ATP by EF_1. Reaction mixtures contained indicated concentrations of sodium phosphate in addition to the components described under Experimental procedures. In acid-quench experiments (unshaded boxes), perchloric acid was added 10 s after initiating the reactions. In cold chase experiments (shaded boxes), excess nonradioactive MgATP was added 10 s after initiating the reactions which was followed 10 s later by the addition of perchloric acid. The details of acid quench and cold chase experiments are described under Experimental procedures.

7% perchloric acid. Radioactive species were analyzed by monitoring labeled ATP, ADP and P_i which were separated by ion-exchange HPLC as described previously [17].

Analytical methods. ATPase activity was assayed with a regenerating system which contained 3 mM ATP, 5 mM Mg^{2+}, 50 mM KCl, 2.0 mM phospho*enol*pyruvate, 5 units of pyruvate kinase, 8 units of lactate dehydrogenase and 0.3 mM NADH in 50 mM Tris-HCl (pH 8.0). Assays were performed at 30 °C.

Reaction mixtures quenched with perchloric acid containing [α,γ-^{32}P]ATP, [α-^{32}P]ADP and [^{32}P]P_i were processed and analyzed for the quantities of radioactive species present in the following manner. The samples were placed in ice for 30 min at which time precipitated protein was removed by centrifugation in a microfuge. The supernatants were neutralized with 2.5 μl of 5 M K_2CO_3, placed in ice for 30 min, and then centrifuged to remove $KClO_4$. The supernatant thus obtained were submitted to anion-exchange HPLC on a TSK DEAE-2SW (0.46 × 25 cm) column that was equilibrated and eluted with 0.40 M sodium phosphate (pH 6.0) at a flow rate of 0.8 ml per min at 23 °C. HPLC was performed with an Alex model 332 liquid chromatograph as described in Ref. 17. Radioactivity was determined with an on-line monitor equipped with 50 μl CsCl solid scintillant cell (Radiomatic Flo-One).

To determine protein removed from centrifuge columns during analysis of enzyme-bound species labeled with ^{32}P, protein pellets obtained after precipitation of enzyme with perchloric acid were resuspended in 200 μl of 5% perchloric acid. The protein was repelleted by centrifugation, freed of supernatant, and then analyzed for protein by the method of Lowry et al. [18]. The recoveries of protein from the centrifuge columns varied from 65 to 85%. Therefore, for each sample, a separate protein determination was performed to calculate the enzyme-bound radioactive species bound to the enzyme.

Computer simulation. A BASIC program using the Runge-Kutta-Gill method was developed for computer simulation of the rate constants of Al-Shawi and Senior [13] and Al-Shawi et al. [14]. The program was run on a NEC PC-9801 computer.

Results

Hydrolysis of substoichiometric [α,γ-^{32}P]ATP by EF_1 in the presence and absence of P_i

Figs. 1A and 2A provide a comparison of the fluxes of radioactive species which develop after mixing EF_1 with [α,γ-^{32}P]ATP in the presence or absence of 2 mM P_i. From this comparison, it is clear that the rate of binding of substoichiometric ATP to EF_1 is diminished significantly in the presence of 2 mM P_i. That P_i inhibits hydrolysis of substoichiometric ATP catalyzed

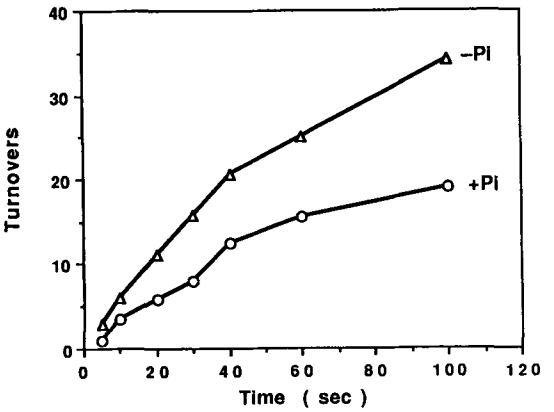

Fig. 4. The effect of 2 mM P_i on the hydrolysis of 1 μM [α,γ-^{32}P]ATP by 1 nM EF_1. Samples of 1 nM EF_1 were incubated with and without 2 mM P_i in 40 mM Mes-SO_4 and 40 mM Tris-SO_4 (pH 7.5) containing 0.25 M sucrose and 0.5 mM $MgSO_4$ for 30 min before adding 1 μl of 50 μM [γ-^{32}P]ATP. A separate reaction mixture was prepared for each time indicated. Completed reactions were quenched by the addition of 10 μl of 14% perchloric acid.

by EF_1 is further illustrated in Fig. 3, which compares the extent of the hydrolysis of 0.3 μM [α,γ-^{32}P]ATP by 1.0 μM EF_1 in the absence of P_i or in the presence of 2 or 50 mM P_i. These results clearly demonstrate that the total amount of ATP committed to hydrolysis in 10 s decreased as the concentration of medium P_i is increased as a consequence of decreased rate of binding of substrate. In contrast, the extent of hydrolysis promoted by the addition of 5 mM nonradioactive ATP is about the same under all conditions as illustrated in Figs. 1A, 2A and 3. However, the magnitude of promoted hydrolysis of radioactive substrate from a preloaded, single catalytic site by excess nonradioactive ATP in these experiments is not nearly as great that observed in comparable experiments with MF_1 [3–5,19]. The relatively weak cold chase observed for EF_1 in this study is also evident in studies reported from the Senior [12–14] and Futai [15,16] laboratories. In parallel with the decreased rate of binding of substoichiometric ATP in the presence of P_i, Fig. 4 shows that 2 mM P_i inhibits hydrolysis of 1 μM ATP by catalytic concentration of EF_1.

The fluxes of enzyme-bound radioactive species formed after adding [α,γ-^{32}P]ATP to EF_1 in the presence or absence of 2 mM P_i, respectively, are illustrated in Figs. 1 B and 2B. Under both conditions and in the absence of promotion the amount of bound [^{32}P]P_i and [α-^{32}P]ADP detected in 20 s is considerably less than the total amount of [α,γ-^{32}P]ATP hydrolyzed at this time. In the absence of P_i, 73% of the [α,γ-^{32}P]ATP added is hydrolyzed in 20 s. However, of the products formed, only 31% of the [^{32}P]P_i and 39% of the [α-^{32}P]ADP remained bound to the enzyme in this interval after passing the samples through centrifuge columns of Sephadex G-50. In the presence of medium P_i, 37% of the added [α,γ-^{32}P]ATP was hy-

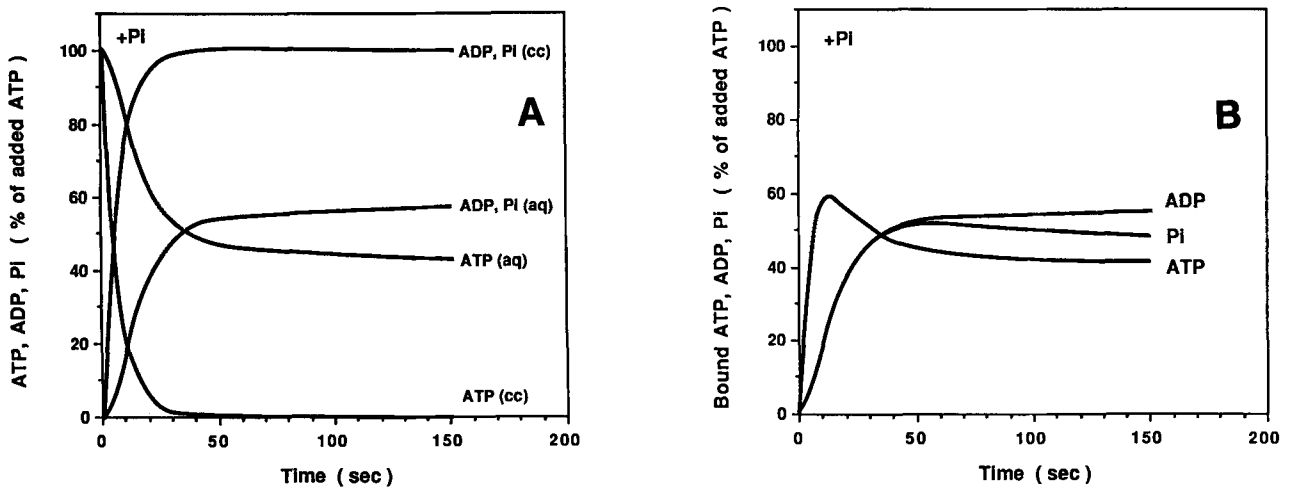

Fig. 5. Computer simulation of hydrolysis of 0.3 μM ATP by 1.0 μM EF_1 using the rate constants reported in Refs. 13 and 14. The initial conditions were set for $[EF_1] = 1.0$ μM and $[ATP] = 0.3$ μM. Integration was performed with a program written in N-88 BASIC using the Runge-Kutta-Gill method. The rate constants used in the simulations were from Al-Shawi and Senior [13] and Al-Shawi et al. [14]. (A) Simulation of fluxes of total species with (cc) and without (aq) cold chase. (B) Simulation of fluxes of bound species in the absence of cold chase.

drolyzed in 20 s. Of the products formed, 34% of the $[^{32}P]P_i$ and 71% of the $[\alpha\text{-}^{32}P]$ADP were tightly bound. Since the amount of bound $[\alpha\text{-}^{32}P]$ADP increased at subsequent sampling times shown in Fig. 1B, part of the $[\alpha\text{-}^{32}P]$ADP bound at 20 s may be the result of released product rebinding to noncatalytic sites.

Computer modelling of published rate constants to simulate hydrolysis of substoichiometric ATP by EF_1.

The experimental results obtained in this study for the hydrolysis of substoichiometric ATP catalyzed by EF_1 are in good agreement with those reported by other laboratories [12–16]. However, computer simulations of fluxes of bound and free species calculated for hydrolysis of substoichiometric ATP by EF_1 from published rate constants [13,14], illustrated in Fig. 5, are inconsistent with the experimental results presented in Fig. 1 and those reported by other laboratories [12–16].

From comparison of the experimental fluxes of substrate and products illustrated in Fig. 1A with the computer simulations of the same fluxes calculated from rate constants illustrated in Fig. 5A, it is clear that the experimentally determined concentrations of ATP, ADP and P_i formed with and without promotion with excess ATP are considerably different from those modelled from the rate constants. Furthermore, the amounts of bound species shown in Fig. 1B are much less than those predicted from simulations presented in Fig. 5B. Computer modelling using the published rate constants predicts that the amount of ATP, ADP, and P_i bound to the enzyme at 150 s, in the absence of promotion, should be 43%, 57% and 50%, respectively, of added ATP. However, the experimentally observed values for bound species are 18% for ATP, 34% for ADP and only 7% for P_i. From the differences between experimentally determined and simulated values for bound ADP, it could be argued that about 40% of the added ATP is hydrolyzed rapidly via bi-site catalysis caused by inadequate mixing. However, when appropriate corrections were incorporated into the computer model to account for 40% rapid, bi-site catalysis *, the amount of P_i observed to be bound to the enzyme experimentally was still considerably less than that predicted from the rate constants. With the corrections, the amounts of ATP, ADP and P_i predicted to be bound to the enzyme by computer simulation are 26, 34 and 30% of the added ATP, respectively. Clearly, computer modelling of the rate constants for uni-site catalysis determined by Al-Shawi et al. [13,14] does not adequately describe the fluxes of substrate and products observed experimentally. Furthermore, when the first-order release of P_i from the single catalytic site is extrapolated to zero time using the rate constant of $1.0 \cdot 10^{-3}$ s^{-1} [13,14], it is estimated that only 14% of the total ATP added to EF_1 under the conditions of Fig. 1 forms P_i which is released with this rate constant. Inorganic phosphate is released from greater than 80% of the enzyme engaged in hydrolysis of substoichiometric ATP at a rate that could not be determined by the method employed. Thus, the rate of P_i release is clearly biphasic.

To assure that these characteristics are not restricted to the experimental conditions of Fig. 1, fluxes of ATP and P_i were computed from published rate

* Initial conditions were set with $[EF_1] = 1$ μM and $[ATP] = 0.18$ μM (60% of 0.3 μM). The remaining 40% of ATP added (0.12 μM) was assumed to be hydrolysed at time zero. The hydrolysis products were added to ADP and P_i detected in hypothetical quench determination. Rebinding of rapidly released product was ignored.

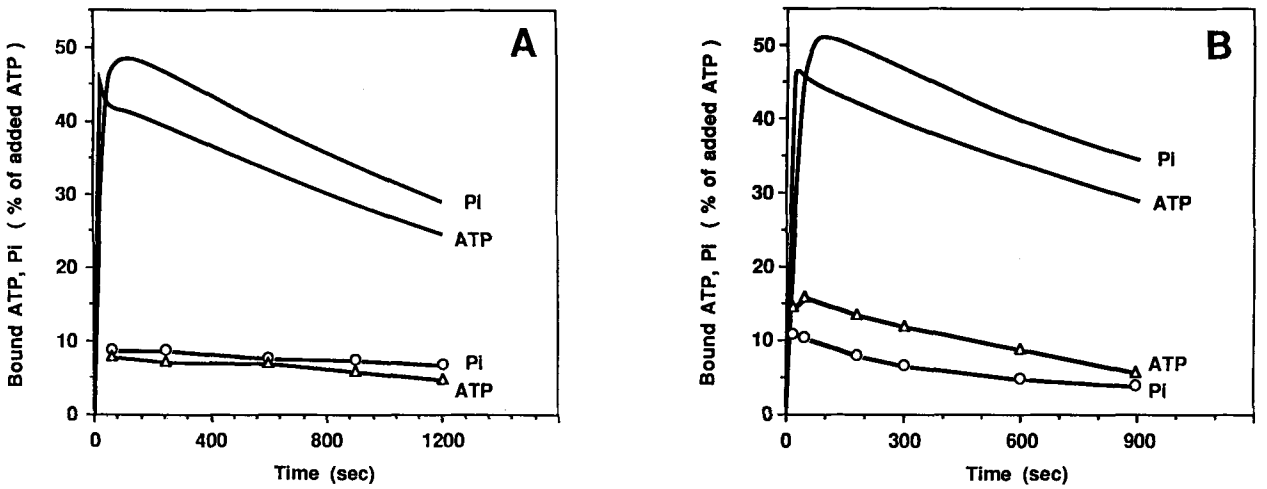

Fig. 6. Comparison of computer simulation for hydrolysis of 1.0 μMATP by 1.0 μM EF_1 and 0.25 μM ATP by 0.5 μM EF_1 with experimental fluxes reported in Ref. 13 and Ref. 15, respectively for those conditions. The algorithm and rate constants are those of Fig. 5. (A) The initial conditions for the simulation were $[EF_1] = [ATP] = 1.0$ μM. The experimental points were taken from the data presented in Fig. 2. of Al-Shawi and Senior [13]. (B) The initial conditions for the simulation were $[EF_1] = 0.5$ μM and $[ATP] = 0.25$ μM. The experimental points were taken from the data presented in Fig. 6 of Noumi et al. [15].

constants to simulate the experimental conditions of Al-Shawi and Senior [13] when they examined hydrolysis of an equivalent amount of [γ-^{32}P]ATP by EF_1. A comparison of computer simulation of bound radioactive species during hydrolysis of 1 μM ATP by 1 μM EF_1 calculated from published rate constants with the experimental results of Al-Shawi and Senior [13] under these conditions are presented in Fig. 6A. It is clear that the experimentally determined, bound species are much lower than the calculated amounts. Fig. 6B shows a comparison of the experimental fluxes of bound ATP and P_i determined by Noumi et al. [15] after adding 0.25 μM [γ-^{32}P]ATP to 0.5 μM EF_1 with computer-simulated fluxes using the rate constants of Al-Shawi and Senior [13]. Again, the experimentally determined bound species are much less than those calculated from the rate constants.

Discussion

It is clear from the results presented in this study and those presented by the Senior [12–14] and Futai [15,16] laboratories that classical features of uni-site catalysis described by Penefsky and his colleagues [3–5] for MF_1 are not exhibited when EF_1 hydrolyzes substoichiometric ATP. At most,only part of the substoichiometric ATP added to EF_1 is hydrolyzed by a mechanism in which bound products in equilibrium with bound substrate are released slowly from a high-affinity site. Instead, the majority of the substoichiometric ATP added to the enzyme binds rapidly and is hydrolyzed with rapid release of products. Clearly, computer simulation of the rate constants of AI-Shawi and Senior, which were determined for the hydrolysis of substoichiometric ATP by EF_1, is not consistent with the experimentally observed fluxes of total and bound species reported here and from the Senior [12–14] and Futai [15,16] laboratories. On the other hand, computer modeling of the rate constants of Grubmeyer et al. [3], as amended by Cunningham and Cross [20], determined for the hydrolysis of substoichiometric ATP by MF_1 are somewhat more consistent with the experimentally observed fluxes of total and bound species reported by Penefsky and his colleagues [3–5].

It might be argued that the observed heterogeneity is caused by extensive bi-site catalysis introduced by inadequate mixing of substoichiometric substrate with the enzyme. This appears not to be the case. Using the same method of manual mixing employed in this study, it was shown in a previous study that an MF_1 preparation hydrolyzed substoichiometric ATP in the presence of 2 mM P_i [19] with characteristics very similar to those observed by Penefsky and his colleagues [3–5]. Since the rate of binding of substoichiometric ATP to EF_1 is only 1% of that observed for the binding of ATP to a single catalytic site of MF_1, it is not likely that significant bi-site catalysis took place during our experiments reported here when 0.3 μM [α,γ-^{32}P]ATP was added to 1 μM EF_1. Supporting this contention, Al-Shawi et al. [14] have presented evidence that bi-site catalysis did not occur during hydrolysis of substoichiometric ATP by EF_1 using another method of manual mixing. The fluxes of bound and free species reported by Al-Shawi et al. [14] are very much similar to those reported here. Although Dunn et al. [21] assumed that biphasic release of product that they observed when examining hydrolysis of substoichiometric ATP by EF_1 in the presence and absence of the ϵ subunit was caused by bi-site catalysis, experimental evidence was not presented to support that this was indeed the case.

As mentioned in Results, computer simulation of the rate constants of Al-Shawi and Senior [13], even with corrections for assumed bi-site catalysis, are inconsistent with the experimental results reported here and by others [12–16,21].

Several factors might be responsible for the heterogeneous hydrolysis of substoichiometric ATP by EF_1 observed in this study and in others [12–16]. Owing to partial loss of minor subunits or partial inactivation, one might argue that the enzyme itself is heterogeneous. However, since our experimental results are in good agreement with those obtained in other laboratories with different preparations of EF_1, enzyme heterogeneity does not appear to cause the heterogeneous kinetics observed. Another possibility is that the observed heterogeneity is related to the endogenous nucleotide content of the enzyme. The EF_1 preparations used by the Futai and Senior laboratories [12–16] contained about 5 mol of adenine nucleotide per mol, which is about the same endogenous nucleotide content of the enzyme preparation used in this study. If it is assumed that 3 mol of adenine nucleotide are bound to noncatalytic sites, then the other 2 mol of endogenous nucleotide would be randomly distributed over three potential catalytic sites, thus causing the heterogeneous kinetics observed. It is clear that a minor fraction of EF_1 hydrolyzes substoichiometric ATP with slow release of products. From this observation, it could be argued that this fraction represents enzyme with three open catalytic sites. However, this possibility is not supported by experiments reported by Hanada et al. [16]. Although they demonstrated that the rate of binding of substoichiometric ATP to EF_1 increased significantly when 3 mol of endogenous nucleotide were removed, the amount of chase promoted hydrolysis observed decreased. This is an enigma. In our view, the factor or factors contributing to the heterogeneous hydrolysis of substoichiometric ATP by EF_1 remain an open question.

The results of this study clearly show that the rate of binding of substoichiometric ATP to EF_1 is inhibited by increasing concentration of P_i. Similar results have been reported by Hanada et al. [16]. According to Penefsky and Grubmeyer [5], MF_1 must be activated with P_i (or ATP) to observe rapid binding of substoichiometric ATP and to overcome a lag observed in hydrolysis of 1 μM ATP by catalytic concentration of the enzyme. In a previous study [19], using an MF_1 preparation with a endogenous nucleotide content different from that used by Penefsky and Grubmeyer [5], the hydrolysis of 1 μM ATP by catalytic concentrations of enzyme proceeded without exhibiting a lag in the absence of activation by P_i. However, incubation of the enzyme with 2 mM P_i stimulated the rate of hydrolysis of 1 μM ATP. In contrast, the hydrolysis of 1 μM ATP by catalytic concentration of EF_1 is inhibited by 2 mM P_i. From this comparison, it is clear that millimolar concentrations of P_i have opposite effects on the capacity of the two enzymes to catalyze both single site and multi-site hydrolysis of ATP.

That the characteristics exhibited when MF_1 hydrolyzes substoichiometric ATP under the conditions described by Penefsky and his colleagues [3–5] may be unique to the mitochondrial enzyme is also suggested by the failure to reproduce these characteristics with other F_1-ATPases. The results presented here show that, at most, only about 15% of the substoichiometric ATP added to EF_1 is hydrolyzed slowly which would be consistent with the mechanism described by Grubmeyer et al. [3] and Cross et al. [4]. As mentioned in the introduction, TF_1 hydrolyzes substoichiometric ATP with rapid release of products. Promotion of hydrolysis by a cold chase was not observed at 23 °C and only a slight amount was observed at 60 °C [10]. Recently, Fromme and Gräber [9] reported that the activated, reduced CF_0F_1 complex from spinach chloroplasts and reconstituted into asolectin liposomes hydrolyzes substoichiometric ATP with much higher rates of release of P_i (0.2 s^{-1}) and ADP (0.1 s^{-1}) than reported for MF_1 [3].

Therefore, of the F_1-ATPases for which hydrolysis of substoichiometric ATP has been examined, slow release of P_i and ADP from the major portion of the single catalytic site is only observed for isolated MF_1 [3–5] or MF_1 in submitochondrial particles activated by removal of its specific inhibitor protein [22,23].

Several mutant forms of EF_1 have been described [12–16,24–26] with substitutions in either the α or β subunit, which show impaired hydrolysis of physiological concentration of substrate, yet retain the capacity to catalyze hydrolysis of substoichiometric ATP added to them that proceeds with slow release of products in a nearly unimpaired manner. It has been suggested that these mutant enzyme forms cannot make the transition from uni-site to multi-site activity when substrate binds to additional catalytic sites. Thus, they are thought to be deficient at sites which are involved in site-site catalytic cooperativity. However, as is observed here and elsewhere [12–16] for the wild-type enzyme, only a small percentage of the substoichiometric ATP added to many of the mutant forms of EF_1 was hydrolyzed with slow release of products. Because of the variety of unknown factors that may impinge on uni-site experiments, there should be more careful attention in the interpretation of these results. Given the lack of universality of hydrolysis of substoichiometric ATP with slow release of products by F_1-ATPases, it is possible that examination of hydrolysis of the major fraction of substoichiometric substrate which occurs with rapid release of products might be more useful in uncovering differences in catalytic properties between wild-type and mutant enzyme forms.

Acknowledgments

We thank Mr. M. Sumi for preparing the figures and manuscript. This work was supported by a grant-in-aid for the scientific area of bioenergetics (to M.Y.) from the Ministry of Education, Science and Culture, Japan, by the National Science Foundation United States–Japan Cooperative Grant INT-87-16615, and by U.S. Public Health Service Grant GM 16,974.

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