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Biochimica et Biophysica Acta 1231 (1995) 275–281

BBA Biochimica et Biophysica Acta

Analysis of time-dependent change of *Escherichia coli* F_1-ATPase activity and its relationship with apparent negative cooperativity

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Received 20 March 1995; accepted 15 May 1995

Abstract

Except for the case of gradual activation of EF_1 (F_1-ATPase from *Escherichia coli*) caused by the dissociation of the ε subunit [Laget, P.P. and Smith, J.B. (1979) Arch. Biochem. Biophys. 197, 83–89], EF_1 has long been thought not to show a time-dependent change in activity [Senior, A.E. et al. (1992) Arch. Biochem. Biophys. 297, 340–344]. Here, we report the time-dependent inactivation and activation of EF_1, which are apparently similar to those of mitochondrial F_1-ATPases [Vasilyeva, E.A. et al. (1982) Biochem. J. 202, 15–23]. Analysis of these changes as a function of ATP concentrations in relation to negative cooperativity revealed that the initial inactivation phase was attributable to the decrease in the V_{max} associated with the low K_m (around 10 μM), and the following activation, probably due to the dissociation of the ε subunit, corresponded to the increase in the V_{max} associated with the high K_m (in the order of 100 μM). Thus, the time-dependent change in EF_1 activity is closely related to the apparent negative cooperativity (multiple K_m values) of ATP hydrolysis.

Keywords: Cooperativity; Kinetics; ATPase, F_1-

1. Introduction

The F_oF_1-ATP synthase of *Escherichia coli* plays an important role in oxidative phosphorylation by coupling transmembraneous proton flow and ATP synthesis [1,2]. F_1 is the water-soluble membrane peripheral part of F_oF_1 and has catalytic sites for ATP synthesis and hydrolysis. Its subunit composition is $\alpha_3\beta_3\gamma\delta\varepsilon$. The F_o portion is a membrane-embedded proton channel. F_1, when isolated from the membrane, catalyzes net ATP hydrolysis and its mechanism has been extensively investigated on the assumption that the hydrolysis is the reverse reaction of synthesis [2]. One of the most prominent characteristics of ATP hydrolysis catalyzed by F_1 is the apparent negative cooperativity [3,4] which is also observed for the membrane-bound enzyme [5,6]. In addition, the F_1-ATPase from bovine heart mitochondria (MF_1) shows an initial inactivation [7–10] and activation during the ATP hydrolysis reaction [11]. These characteristics are thought to reflect interactions between multiple catalytic sites or noncatalytic sites and catalytic sites [11,12]. However, such an inactivation or activation has not been reported in the case of the F_1 from *E. coli* (EF_1), except for the case of the activation caused by the dissociation of the ε subunit from the enzyme molecule [13–15].

In the present study, we demonstrate that EF_1 also exhibits the time-dependent initial inactivation followed by slow activation like MF_1. We analyzed the time-dependent change in ATPase activity in relation to the apparent negative cooperativity (expressed by multiple K_m values) and found that they are closely related to each other. The initial inactivation corresponded to the decrease in the V_{max} associated with the low K_m (around 10 μM), and the following activation, which is most likely caused by the dissociation of the ε subunit, corresponded to the increase in the V_{max} associated with the high K_m (in the order of 100 μM).

Abbreviations: EF_1, F_1-ATPase from *Escherichia coli*; MF_1, F_1-ATPase from mitochondria; HPLC, high-performance liquid chromatography; DTT, dithiothreitol; nd-EF_1, EF_1 depleted of nucleotides.

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SSDI 0005-2728(95)00087-9

2. Materials and methods

2.1. Preparation of native EF_1 and nd-EF_1

Native EF_1 was prepared according to Wise [16] with some modifications as follows. After solublization, the crude EF_1 fraction was directly applied on a DEAE-Toyopearl ion-exchange column (Tosoh, Japan). Peak protein fractions were collected and precipitated with 65% saturated ammonium sulfate. The enzyme was then further purified with a G3000SW gel-filtration HPLC column (Tosoh, Japan) equilibrated and eluted (flow rate: 1 ml/min.) with 50 mM Tris-HCl (pH 7.4), 10% (v/v) methanol, 40 mM 6-aminohexanoic acid, 2 mM EDTA, 1 mM ATP, 1 mM DTT and 150 mM Na_2SO_4. Peak protein fractions were collected and precipitated with 65% saturated ammonium sulfate. Purified EF_1 (precipitant) was dissolved in the storage buffer consisting of 50 mM Tris-H_2SO_4 (pH 7.5), 2 mM EDTA, 1 mM DTT, 40 mM 6-aminohexanoic acid, 10% (v/v) glycerol and 1 mM ATP, and stored at −20°C. The specific activity at 37°C and 5 mM of ATP was about 80 U/mg. EF_1 depleted of nucleotides (nd-EF_1) was prepared according to Senior et al. [17]. The nd-EF_1 was stored at room temperature in 100 mM Tris-H_2SO_4 (pH 8.0) containing 4 mM EDTA and 50% (v/v) glycerol. The specific activity was about 75 U/mg at 37°C. The amounts of bound nucleotides were analyzed by HPLC [18]. Native EF_1 used in this study contained about 1.4 mol of ADP/mol of enzyme and 0.6 mol of ATP/mol of enzyme. nd-EF_1 contained about 0.1 mol of ADP/mol of enzyme and essentially no ATP. Protein concentration was measured by the method of Bradford [19].

2.2. Measurement of ATPase activity

The ATPase activity of EF_1 was measured spectrophotometrically using an ATP regenerating system [20] at 25°C with the spectrophotometer UV-2200 (Shimadzu, Japan). Before measurement of ATPase activity, EF_1 was passed through a 1 ml of Sephadex G-50 fine centrifuge column [21] twice to remove ATP in the storage buffer. In the case of nd-EF_1, there is no ATP in the sample buffer, and this step was omitted. The enzyme was then diluted to about 0.2 μM in a buffer containing 50 mM Tris-H_2SO_4 (pH 8.0), 2 mM EDTA, 1 mM DTT, 40 mM 6-aminohexanoic acid and 10% (v/v) glycerol to avoid irreversible inactivation during the experiment. The basic assay medium consisted of 50 mM Tris-H_2SO_4 (pH 8.0), 10 mM KCl, 2.5 mM phospho*enol*pyruvate, 0.15–0.3 mM NADH, 50 μg/ml pyruvate kinase, 50 μg/ml lactate dehydrogenase (both enzymes were obtained from Boehringer in 50% glycerol) and 2 mM $MgSO_4$. In a preliminary experiment, we tested different amounts of coupling enzymes in the assay mixture and confirmed that the above condition is sufficient to follow the ATPase activity within the time scale employed here. When a buffer system containing chloride instead of sulfate was employed, qualitatively the same results as shown in Results were obtained. When the ATPase reaction was started by the addition of EF_1 to the basic assay medium (F_1-started), various concentrations of Mg-ATP (1–5000 μM) were added to the basic assay medium before the EF_1 addition. The reaction was then initiated by the addition of EF_1 solution to a final concentration of about 3 nM. The time-course of the data was collected every 0.5 s for 630 s and stored in an on-line computer. When the ATPase reaction was started by the addition of ATP (ATP-started), EF_1 was added to the assay medium at a concentration of about 3 nM and previously incubated at 37°C for at least 15 min to complete the activation. (At 25°C, the activation took about 1 h in the absence of adenine nucleotides (data not shown).) The assay medium was then cooled to 25°C and the reaction was initiated by the addition of stock ATP-Mg solution. The stock ATP-Mg solution was prepared by mixing the basic assay medium containing the ATP regenerating system with concentrated ATP-Mg solution to remove the contaminated ADP. Final ATP concentrations were varied from 1 μM to 1500 μM. One unit of enzyme activity was defined as that producing 1 μmol of ADP per minute.

3. Results

3.1. ATP hydrolysis initiated by the addition of EF_1

Typical time-courses of ATP hydrolysis are shown in Fig. 1. The reaction was initiated by the addition of F_1 (F_1-started). Fig. 1A shows the traces of the absorbance change at 340 nm. The original traces were converted to their derivative over time (dA_{340}/dt) to show more clearly the changes in the activities (Fig. 1B) where the decrease in dA_{340}/dt is representative of activation of ATPase and the increase is representative of inactivation. At high concentrations of ATP-Mg (above 300 μM), a significant activation phase (Fig. 1A and B, trace a) was observed. This is similar to the activation reported by Laget and Smith [13] and is likely to be caused by the dissociation of the ε subunit from the enzyme. The rate of activation was estimated by fitting an exponential curve to the derivatised data. The rate of activation depended on the enzyme concentration, which is consistent with the hypothesis of dissociation of the ε subunit from EF_1, but it did not depend on the ATP concentration when ATP concentration was above 300 μM. (Under 300 μM of ATP, we could not estimate the rate of activation because of the overlapping of the preceding inactivation phase (data not shown).) At intermediate concentrations of ATP (between 50 μM and 300 μM), the time-courses consisted of three kinetic phases; an initial inactivation phase (increase in dA_{340}/dt), a slow activation phase (decrease in dA_{340}/dt), and a final, steady state (constant negative value of dA_{340}/dt)

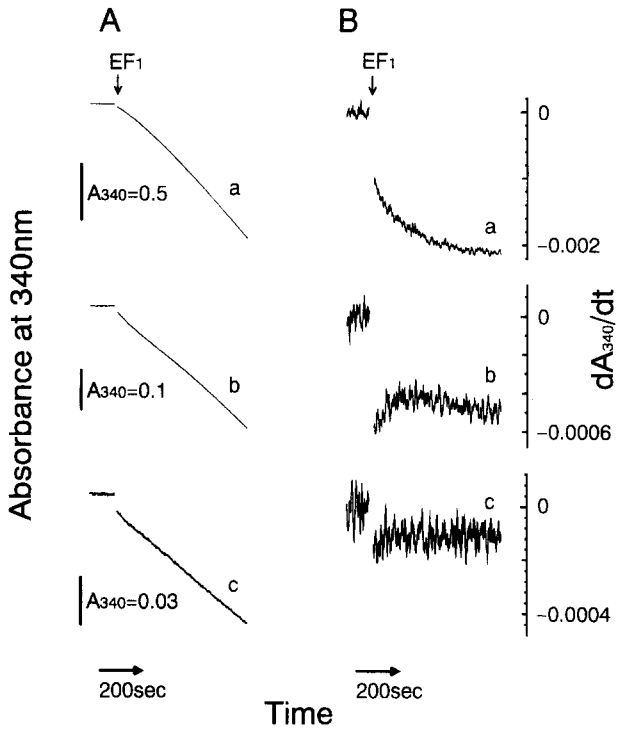

Fig. 1. Time-courses of ATP hydrolysis by nd-EF_1 with F_1-started reaction. The changes in absorbance at 340 nm are shown in (A) (left column). ATP concentrations were 3.7 mM (trace a), 57 μM (trace b) and 4.4 μM (trace c). In this case, reaction was initiated by the addition of EF_1 at the time indicated by the arrow (F_1-started). The derivatives of A over time (dA_{340}/dt) are drawn in (B) (right column).

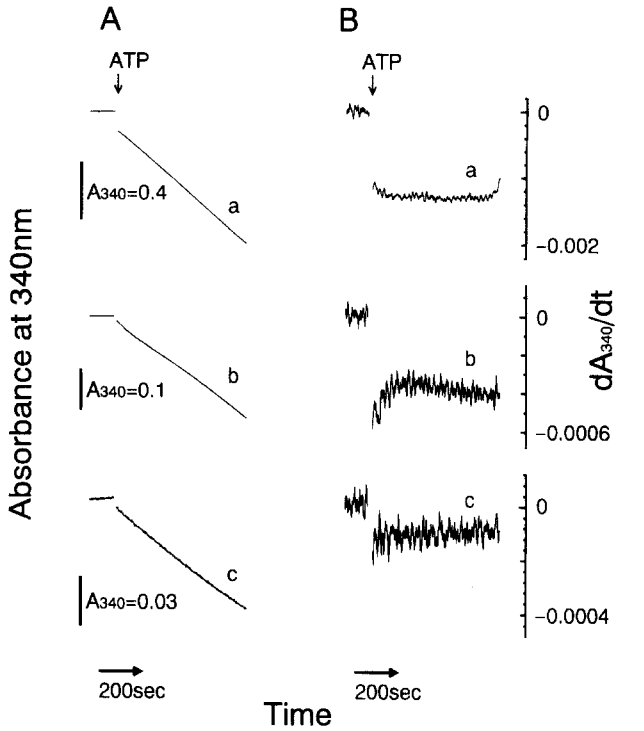

Fig. 2. Time-courses of ATP hydrolysis by nd-EF_1 with ATP-started reaction. In this case, reaction was initiated by the addition of ATP at the time indicated (ATP-started). ATP concentrations were 1.5 mM (trace a), 75 μM (trace b) and 7.5 μM (trace c). (A) and (B) are the same form as Fig. 1.

(Fig. 1A and B, trace b). The activation phase disappeared (Fig. 1A and B, trace c) at low concentrations of ATP (between 5 μM and 50 μM), and below this concentration range, there was no apparent time dependency. Results for native EF_1 and nd-EF_1 were identical (data not shown), and therefore further experiments were carried out with nd-EF_1.

3.2. ATP hydrolysis initiated by the addition of ATP

As stated above, the activation phase corresponds probably to the dissociation of the ε subunit from the enzyme [13], however, the initial inactivation observed at relatively low ATP concentrations has not been reported for EF_1 before. In order to characterize these time-dependent changes, it would be desirable to separate the inactivation phase from the activation one. Because the activation is induced by the dissociation of the ε subunit and the K_d of the ε and EF_1 is in the order of nM [13], EF_1 is spontaneously activated even in the absence of ATP when it is diluted to the nM range. In a preliminary experiment, we found that the activation of EF_1 by the dilution took about 1 h at 25°C but less than 15 min at 37°C in the absence of ATP (data not shown). Thus, we first incubated the enzyme in the assay medium at a concentration of about 3 nM at 37°C for 15 min to let the ε subunit dissociate from

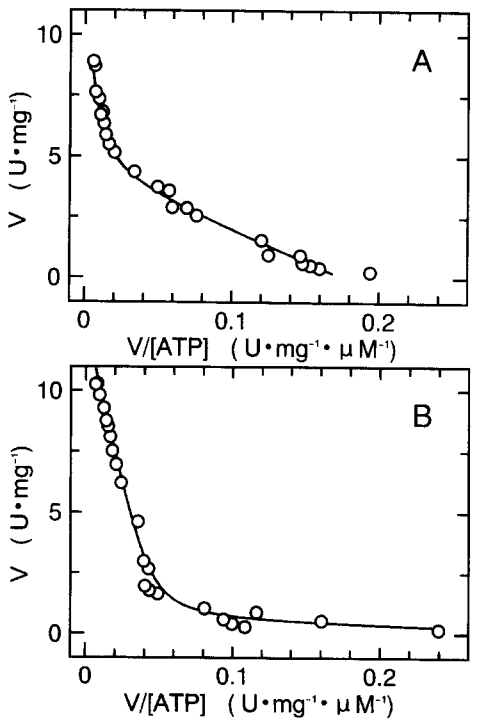

Fig. 3. Typical Eadie-Hofstee plots of ATP hydrolytic activity of nd-EF_1. Data represented here are obtained from the ATP-started reaction. Solid lines are theoretical curves from determined kinetic parameters (see Fig. 5). Velocity data of (A) and (B) were obtained from the velocities at 30 s and 430 s after the initiation of the reaction, respectively.

EF_1. The assay medium was then cooled down to 25°C, and the reaction was initiated by the addition of stock ATP-Mg solution (ATP-started). Typical results of ATP-started reaction are shown in Fig. 2. (A represents the original data, and B is the derivative). The activation phase at high ATP concentration observed in the F_1-started reaction almost disappeared in the ATP-started reaction (compare trace a of Fig. 1A, B and Fig. 2A, B). However, there was still slight activation in the middle range of ATP concentration (Fig. 2A, B trace b). In contrast, the initial inactivation phase remained the same as that of the F_1-started reaction (traces b, c). We tried to judge whether or not the rate of inactivation depends on ATP concentration. However, due to the following slight activation and the insufficient quality of the data, we could not find any clear tendency.

3.3. Time-dependent change in the kinetic parameters

The observation described above clearly showed that the inactivation and the activation shown at high ATP concentration were independent. Because the initial inactivation and activation phases occurred with different dependencies for ATP concentration at different time-scales, these changes were expected to affect the cooperative kinetics of ATP hydrolysis. In order to examine the time-dependent change in the kinetic parameters of ATP hydrolysis, the rate of ATP hydrolysis was calculated by least-square linear fitting on the time-course at every 50 s. From these velocity data obtained at various ATP concentrations, Eadie-Hofstee plots were constructed at various times from the start of reaction. Typical plots are shown in Fig. 3. It is clear that the shape of the Eadie-Hofstee plots change with time. In order to analyze these data, we assumed a kinetic model as shown in Scheme 1. In the ATP concentration range examined in this study, the high affinity single catalytic site is always saturated [22–24] and only the participation of the second and third catalytic sites is taken into account. (See Appendix A for details.) The kinetic parameters were determined by non-linear regression methods. The reliabilities of the parameters were checked

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P \quad (1)$$

$$ES + S \underset{k_5}{\overset{k_4}{\rightleftharpoons}} ESS \xrightarrow{k_6} ES + P \quad (2)$$

Scheme 1. Reaction scheme which represents negative cooperativity of ATP hydrolysis by EF_1. E, S, P, ES and ESS represent the free EF_1, ATP, ADP plus P_i, EF_1-ATP complex and EF_1-2ATP complex, respectively. In this scheme, the reaction mainly proceeds as (1) in low ATP concentration and mainly as (2) in high ATP concentration. In the middle range of ATP concentration, the reaction proceeds via both (1) and (2). When K_m and V_{max} are defined as below, velocity v can written as follows. $K_{m1} = (k_2 + k_3)/k_1$, $V_{max1} = k_3 \cdot [Et]$, $K_{m2} = (k_5 + k_6)/k_4$, $V_{max2} = k_6 \cdot [Et]$, $v = ([S]^2 V_{max2} + [S] V_{max1} \cdot K_{m2})/([S]^2 + [S] K_{m2} + K_{m1} \cdot K_{m2})$ See Appendix A for details.

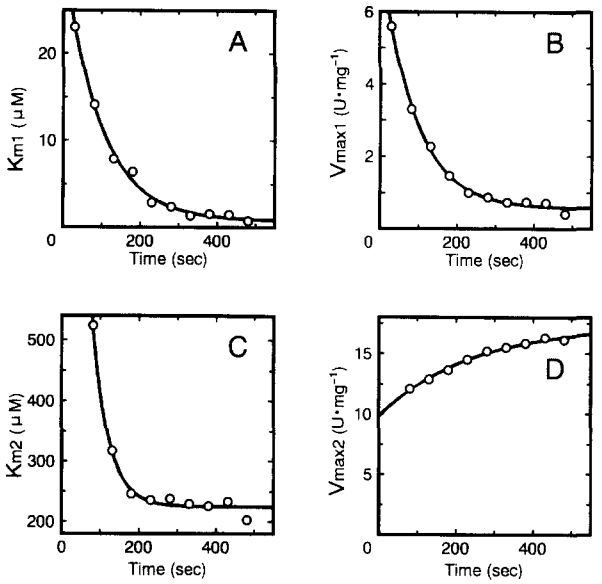

Fig. 4. Time-dependent change in the kinetic parameters for the F_1-started reaction. Panels (A), (B), (C) and (D) represent time-dependent changes in K_{m1}, V_{max1}, K_{m2}, and V_{max2}, respectively. These values change with time as apparent first-order reactions. Solid lines are single exponential curves determined from least-square curve fitting methods. Apparent first order rate constants for the changes in K_{m1}, V_{max1}, K_{m2} and V_{max2} are $1.06 \cdot 10^{-2}$ s^{-1}, $1.14 \cdot 10^{-2}$ s^{-1}, $2.38 \cdot 10^{-2}$ s^{-1} and $4.20 \cdot 10^{-3}$ s^{-1}, respectively.

by superimposing theoretical curves on experimental data in the substrate-velocity plot and Eadie-Hofstee plot (Fig. 3, solid lines). When these parameters were plotted against time, they showed characteristic time-dependencies as

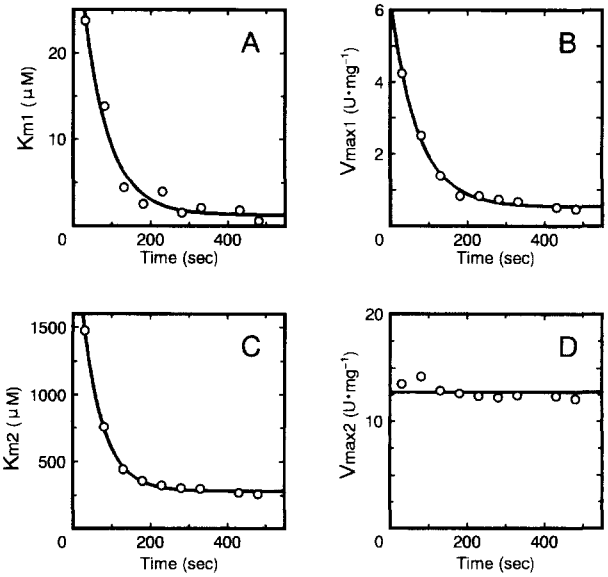

Fig. 5. Time-dependent change in the kinetic parameters with ATP-started reaction. The form of the figure is the same as Fig. 4. K_{m1}, V_{max1}, and K_{m2} changes as apparent first-order reactions with rate constants of $1.52 \cdot 10^{-2}$ s^{-1}, $1.39 \cdot 10^{-2}$ s^{-1}, and $1.88 \cdot 10^{-2}$ s^{-1}, respectively, while V_{max2} remained constant (12.8 ± 0.7 U / mg).

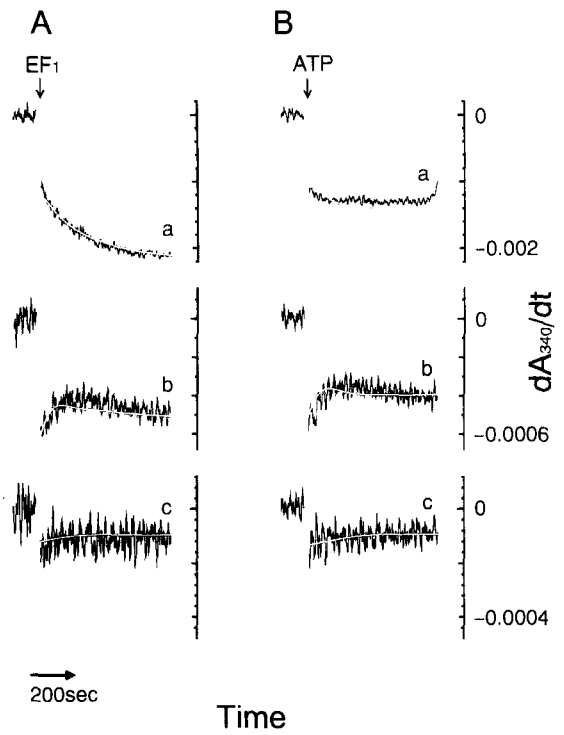

Fig. 6. Comparison of time-courses of ATPase activity of nd-EF_1 and theoretical lines. Time-courses of ATPase activity of nd-EF_1 were calculated by kinetic parameters determined with Scheme 1. (A) F_1-started reaction. Traces a, b and c are same as Fig. 1B. (B) ATP-started reaction. Traces a, b and c are same as Fig. 2B. Black lines represent experimental data, and white lines represent reproduced time-courses.

shown in Fig. 4 (F_1-started) and Fig. 5 (ATP-started). Except for the V_{max2} in the ATP-started reaction (Fig. 5D), these values changed as apparent first-order reactions, and the rate constant of the time-dependent change for each parameter was calculated by fitting with an exponential curve. The effect of the activation caused by the dissociation of the ε subunit is clearly observed in the different time dependency of V_{max2} between F_1-started and ATP-started reactions (Fig. 4D and 5D). In Fig. 4D, V_{max2} increases with time, reflecting the activation caused by the dissociation of the ε subunit. On the other hand, when the reaction was initiated after the dissociation of the ε subunit, V_{max2} remained essentially constant (Fig. 5D) In contrast to the activation phase, the initial inactivation is attributable to the decrease in V_{max1} for both types of reaction (Fig. 4B and 5B), which is about 3 times faster than the increase in V_{max2} (Fig. 4B and 4D). To check the validity of these analyses, the time-courses of ATPase activity were calculated from the obtained time-dependent kinetic parameters and superimposed on experimental data. They qualitatively reproduced the time-courses of the ATPase activity (Fig. 6). Thus, the complex time-dependent change in ATPase activity can be explained by a combination of independent changes in V_{max} values associated with high and low K_m values.

4. Discussion

In the present study, we have shown that the ATPase activity of EF_1 undergoes time-dependent inactivation and activation phases. When the ATPase reaction is initiated by the addition of enzyme (F_1-started, Fig. 1), activation proceeds on a time scale of minutes. The time-dependent activation almost disappeared when the enzyme was first diluted and the ATPase reaction was initiated by the addition of ATP (ATP-started, Fig. 2). These characteristics agree well with those reported by Laget and Smith [13], and we conclude that this activation is mainly caused by the dissociation of the inhibitory ε subunit from the enzyme as previously proposed [13,15]. The extent of activation observed in this study (about 1.5-fold) was not as high as that in the previous report (about 4-fold, [13]). This may be due to some dissociation of the ε subunit during enzyme preparation or differences in the assay conditions. Some slight activation was still observed when the ATPase reaction was initiated by the addition of ATP (Fig. 2B, a and b). This may be caused by binding of ATP to a non-catalytic site as proposed for nd-MF_1 [11] or reflect incomplete dissociation of the ε subunit during pre-incubation of the enzyme. In the case of EF_1, however, the native enzyme containing 2 bound nucleotides and the nucleotide-depleted enzyme gave identical results. This activation was so slight that we did not further examine it precisely.

The inactivation phase of the ATPase activity of EF_1 was demonstrated for the first time by derivatizing the time-course data. The reason why there has been no report for the time-dependent inactivation may lie in some differences in assay conditions such as different free Mg^{2+} concentrations, temperature or the method of determination of hydrolysis in previous studies. In fact, when the free Mg^{2+} concentration was lower than 1 mM, or the assay was carried out at 37°C, the initial inactivation phase was not so evident (data not shown).

The most interesting finding in the present study is that the time-dependent changes in ATPase activity are closely related to the apparent cooperative kinetics of the ATPase activity of EF_1. When the ATPase activity was defined at every 50 s after initiation of the reaction and Eadie-Hofstee plots were constructed, the shape of the plots changed with time (Fig. 3). Analysis of the data of the F_1-started reaction according to Scheme 1 revealed that the initial inactivation phase corresponded to the decrease in V_{max} (V_{max1}) associated with low K_m (K_{m1}) and the following activation phase corresponded to the increase in V_{max} (V_{max2}) associated with high K_m (K_{m2}) (Fig. 4). The larger rate constant of the change in V_{max1} than that of V_{max2} reflects the fact that inactivation precedes activation. When the data for the ATP-started reaction was analyzed, V_{max1} decreased as in the F_1-started reaction while V_{max2} was essentially constant (Fig. 5), which is consistent with the results that slow activation was almost absent in the

ATP-started reaction (Fig. 2). These results strongly suggest that the different kinetic modes (multiple K_m values) deduced by curve fitting actually reflect some different kinetic pathways of the ATPase reaction which can change independently.

As stated above, the increase in V_{max2} is mainly attributable to the dissociation of the ε subunit from the enzyme. On the other hand, the cause of the decrease in V_{max1} is not clear in the present study, but it may be related to the inhibition caused by ADP-Mg at a catalytic site [9]. In fact, pre-incubation of EF_1 with ADP-Mg causes an inhibitory effect on the ATPase activity and the initial inactivation becomes obscure when the free Mg^{2+} concentration is lowered (data not shown). Recently Hyndman et al. [25] reported ADP-Mg inhibition of EF_1 under a quite different condition (i.e., very low concentration of ATP (50 nM)). Direct comparison of the present results with those obtained under different conditions or with other F_1's may be difficult, but it seems also related to the report that the inhibitory effect of NaN_3, which is supposed to be linked to ADP-Mg inhibition, was related to the low K_m mode of ATP hydrolysis by submitochondrial particles and isolated mitochondrial F_1-ATPase [26].

Continuous changes in the V_{max} values may be explained as the results of the dissociation of the ε subunit (V_{max2}) and ADP-Mg inhibition (V_{max1}). However, it is not apparent why the K_m value changed continuously with time because conformational changes in the enzymes are generally supposed to occur between some distinct states. As for the change in K_{m1}, however, it can be explained by Scheme 2, which assumes a slow transition between the active ES complex and the inactive (or less active) ED complex. In this case, it is necessary to assume that the ED complex is still active in making the EDS complex and catalyzing the hydrolysis of ATP in the high K_m (K_{m2}) mode because V_{max2} remains essentially constant in the ATP-started reaction. As for the change in K_{m2}, we could not find a good explanation. However, the slight activation

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P \quad (1)$$

$$ES + S \underset{k_5}{\overset{k_4}{\rightleftharpoons}} ESS \xrightarrow{k_6} ES + P \quad (2)$$

$$ED + S \underset{k_5}{\overset{k_4}{\rightleftharpoons}} EDS \xrightarrow{k_6} ED + P \quad (3)$$

Scheme 2. Formation of inactive complex during ATP hydrolysis reaction. This scheme was derived from Scheme 1 with an additional inactivation pathway. During the ATPase reaction with the low K_m site, the inactive ED complex is formed at a very slow rate k_7 and recovered at rate k_8. The ED complex is inactive in reaction (1) but still active for the high K_m mode as reaction (3) (same as reaction (2)). See Appendix B for details.

which we have ignored may affect it. Whether or not the rates of activation or inactivation depend on the ATP concentration is of interest, however, as stated in the Results section, we could not judge this point due to the insufficient quality of the data. Obviously, further rigorous analyses are required to elucidate the feature of the time-dependent change in the kinetics of EF_1.

In summary, we demonstrated the existence of the initial inactivation and successive activation phase of the ATPase activity of EF_1 and found their relationship to the apparent negative cooperativity. Further precise analyses will provide insights into the mechanism of complex kinetic behavior of this enzyme.

Acknowledgements

We thank Dr. Jean-Michel Jault for his keen interest in our work and insightful discussions and Dr. Toru Hisabori for providing us the data analysis tool, 'S.C.'.

Appendix A

Explanation for Scheme 1

Usually, the apparent negative cooperativity of F_1-ATPase is analyzed as a sum of two or three independent Michaelis-Menten reactions [3–5]. The kinetic parameters assuming two independent Michaelis-Menten type reactions can be converted to those from Scheme 1 in the present study as follows,

$$K_{m1} = K_{M1} \cdot K_{M2}/(K_{M1} + K_{M2})$$

$$V_{max1} = (V_{MAX1} \cdot K_{M2} + V_{MAX2} \cdot K_{M1})/(K_{M1} + K_{M2})$$

$$K_{m2} = K_{M1} + K_{M2}$$

$$V_{max2} = V_{MAX1} + V_{MAX2}$$

Here the left columns are the parameters from Scheme 1 and the right columns (capitalized) are the parameters from two independent Michaelis-Menten type reactions. The same conclusion was derived when the data were analyzed as a sum of two Michaelis-Menten type reactions. The quality of the fit was not improved when we assumed a sum of three Michaelis-Menten reactions.

We favor Scheme 1 because it can be adopted to negative, positive and no cooperativity by choosing the appropriate set of parameters. The relationship between the range of kinetic parameters and the apparent cooperativity is summarized as follows.

Under the condition of $V_{max1} < V_{max2}$ (substrate inhibition does not occur), and when the parameter C is defined as below, the apparent cooperativity is given by the sign of C.

$$C = V_{max1} \cdot K_{m2}(V_{max1} - V_{max2}) + V_{max2}^2 \cdot K_{m1}$$

$C > 0$;apparent positive cooperativity.

$C = 0$;no cooperativity.

$C < 0$;apparent negative cooperativity.

Appendix B

Explanation for the change in K_{m1} and V_{max1}

The change in the kinetic parameters of the low-K_m mode (K_{m1}, V_{max1}) may be explained by an idea that the inactivation is caused by the formation of an inactive ED complex from the ES complex as shown in Scheme 2. There are at least two possible explanations for the continuous changes in K_{m1} and V_{max1} based on this scheme.

The first possibility is that the rate of formation of the inactive complex (written as ED in Scheme 2) and the final equilibrium between ES and ED depends on the substrate concentration (k_7 in Scheme 2 contains [S] as $k_7 = k'_7$ [S]). In this case, at higher substrate concentration, formation of the inactive complex is faster and occurs to a greater extent. This will result in higher suppression of ATPase activity at higher substrate concentration, and the substrate concentration which gives half-maximum activity will continuously decrease as inactivation proceeds with time. In this case, the rate of inactivation will depend on the ATP concentration.

The second possibility assumes a very slow equilibrium between ES and ED, which is independent of substrate concentration (k_7 in Scheme 2 does not contain [S]). During the reaction process, ES becomes ED at a very slow rate, k_7. The ratio of ES to ES + ED (ES/(ES + ED)) increases with time. Apparent K_{m1} and V_{max1} derived from Scheme 2 appear as (ES/(ES + ED)) $K_{m1,0}$ and (ES/(ES + ED)) $V_{max1,0}$, respectively ($K_{m1,0}$ and $V_{max1,0}$ are values without the formation of ED (ES/(ES + ED) = 1)). In this case, the rate of inactivation will not depend on the ATP concentration but K_{m1} and V_{max1} decrease continuously with time. The first possibility is one of the variations of the second one. Similar rates of the changes in V_{max1} and K_{m1} are consistent with these ideas.

Thus, these two possibilities can be distinguished by the ATP concentration dependency of the inactivation rate. However, due to the following slight activation phase and insufficient quality of the raw data, we could not judge whether the rate of inactivation depends on ATP concentration or not.

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