Biochimica et Biophysica Acta, 589 (1980) 241—255

BBA 47809

THE PRE-STEADY STATE AND STEADY-STATE KINETICS OF THE ATPase ACTIVITY OF MITOCHONDRIAL F_1

O.A. ROVERI *, J.L.M. MULLER, J. WILMS and E.C. SLATER

Laboratory of Biochemistry, B.C.P. Jansen Institute, University of Amsterdam, Plantage Muidergracht 12, 1018 TV Amsterdam (The Netherlands)

(Received July 13th, 1979)

Key words: Mitochondrial ATPase; F_1-ATPase; Kinetics

Summary

1. The lag time before maximum velocity of ATP hydrolysis is reached upon mixing ATP with F_1 is much greater than can be explained by a simple Michaelis-Menten mechanism, and must be due to an activation reaction. The lag time is dependent on the concentration of MgATP (half-maximal at 30 μM) and is equal to 30 ms at infinite MgATP concentration. The initial rate of hydrolysis by nucleotide-depleted F_1 is much greater than with normal F_1. It is tentatively suggested that the activation reaction with normal preparations is due to replacement of firmly bound ADP by MgATP.

2. After the initial time lag, the reaction follows very closely first-order kinetics provided that the concentration of MgATP is much less than the K_m and the reaction is completed within 2 s. This is not expected if the dissociation constant of the enzyme-MgADP complex, an intermediate in the enzymic reaction, is much lower than the K_m as has been reported in the literature. The value of V/K_m, calculated from the exponential decay, is very close to that calculated from independent measurements of V and K_m.

3. The low values for K_i(ADP) reported in the literature were found to be due to a slow (in the order of seconds) formation of an inhibited MgADP-enzyme complex. Dissipation of this inhibited complex by ATP requires seconds. The dissociation constant of the MgADP-enzyme complex that is an intermediate in the enzyme reaction was found to be 150 μM.

4. ADP but not ATP becomes firmly bound to nucleotide-depleted F_1 in the absence of Mg^{2+}.

* Present address: Centro de estudios fotosintéticos y Bioquímicos (Consejo Nacional de Investigaciones, Fundacíon M. Lillo, Universidad Nacional de Rosario), Suipacha 531, 2000 Rosario, Argentina.

Introduction

It is widely accepted that the terminal reaction of oxidative phosphorylation, the synthesis of ATP from ADP and P_i, is catalysed by the mitochondrial ATPase F_1, first extracted and purified by Racker and coworkers [1]. Although the isolated enzyme catalyses only the reverse reaction, the hydrolysis of ATP, it is highly likely that the catalytic centre involved in this reaction is also involved in the synthesis. Studies of the mechanism of hydrolysis of ATP are, therefore, relevant for that of its synthesis.

Despite its importance, we know much less about the mechanism of this enzyme than that of, for example, myosin. In the latter case the kinetic and equilibrium constants of the partial reactions of a simple sequential mechanism involving a single catalytic site and 6 intermediates are for a large part known [2]. Of particular interest is the fact that the dephosphorylation of myosin-bound ATP to ADP and P_i, both still bound to the protein, proceeds with little change of Gibbs energy and is demonstrably reversible. Boyer [3] and Slater [4] have postulated that the same is true for F_1, but the necessary evidence for or against this proposal from kinetic studies is lacking.

Recently we have proposed that F_1 contains 7 ADP- and/or ATP-binding sites, classified into 4 types [5]. Wagenvoord et al. [6,7] have brought forward evidence that 2 of these sites (Type III [5]), one on each of the two β-subunits [8], are catalytic sites, which in the ATPase reaction at least act independently of one another. It is postulated that the other sites have a regulatory function [5]. This has been shown directly to be the case for an anion-binding site that can also bind ATP [9,10]. It has been suggested [5] that there are two such sites, one on each of the α-subunits.

In the present paper it is shown that three phases in the course of the hydrolysis of ATP may be distinguished: a lag phase much longer than can be accounted for by formation of the Michaelis-Menten intermediate by reaction between free enzyme and ATP, a steady-state phase, and an inhibitory phase due to the slow formation of an inhibited ADP-enzyme complex. The results give further confirmation of the presence of regulatory sites, but, although they reveal a close resemblance between the mechanism of myosin and F_1 ATPase, they give no information on the question whether, like myosin, the dephosphorylation-phosphorylation reaction between enzyme-bound components is close to equilibrium.

Materials and Methods

F_1 was prepared according to Knowles and Penefsky [11] and taken up in a solution containing 250 mM sucrose, 10 mM Tris, 2 mM EDTA and 4 mM ATP. The pH was brought to 7.5 with acetic acid or H_2SO_4. Samples were stored in liquid nitrogen. After thawing, the protein was washed three times by precipitation with 50% satd. $(NH_4)_2SO_4$ and dialysed for 3—4 h, changing the buffer each hour, against a buffer that will be specified in the legends. After the dialysis the specific activity of the preparation was 100—130 $\mu mol \cdot min^{-1} \cdot mg^{-1}$, measured as described by Wagenvoord et al. [6] but in the presence of 10 mM HCO_3^-.

Preparation of nucleotide-depleted F_1

F_1 was treated by the method of Garrett and Penefsky [12] with some modifications. The enzyme, washed three times by ammonium sulphate precipitation, was suspended in a buffer containing 50% glycerol, 50 mM Tris-H_2SO_4 buffer and 10 mM sodium pyrophosphate at pH 8.0, and dialysed against the same medium for 3 days, twice replacing the dialysis buffer. In order to remove the pyrophosphate the preparation was then dialysed for 4 days against the glycerol buffer in which the pyrophosphate was omitted. All operations were carried out at about 20°C. After this treatment, the enzyme was stable for several weeks at this temperature. The specific activity was 140—150 $\mu mol \cdot min^{-1} \cdot mg^{-1}$. The preparation contained less than 0.6 mol ATP and less than 0.2 mol ADP per mol enzyme, as measured by the method of Harris et al. [13].

Determination of the release of protons during the ATP hydrolysis

The release of protons that accompanies the hydrolysis of ATP was followed spectrophotometrically in a Durrum stopped-flow apparatus, by means of a pH indicator, essentially as described by Bagshaw and Trentham [14]. Phenol red (pK 7.9) was used, the pH changes being followed at 560 nm (ΔA = 54 $cm^{-1} \cdot mM^{-1}$). The solutions were depleted of CO_2 with a vacuum pump and the pH was adjusted with NaOH under a flow of CO_2-free N_2. The buffer capacity was determined by measuring the changes in absorbance, with an Aminco-Chance DW-2 at 560 nm, produced by the addition under N_2 of known amounts of oxalic acid. The H^+/ATP ratio was assumed to be 1.0 at pH 8.0. The maximal change in the pH during the experiments was less than 0.1 unit different from the pK of the indicator, in which region there is a linear relationship between the absorbance and the proton concentration.

Determination of the inorganic phosphate production during ATP hydrolysis

In some experiments ATP hydrolysis was followed by measuring the appearance of $^{32}P_i$ from [γ-^{32}P]ATP. The reaction was stopped by the addition of $HClO_4$ and after centrifugation 0.10 ml 12 M HCl, 0.85 ml water, and 0.75 ml 60 mM ammonium molybdate in 0.1 M HCl were added to 0.8 ml supernatant. The phosphomolybdate complex was extracted by 4 ml isobutanol/benzene (50 : 50, v/v) saturated with water. The $^{32}P_i$ was determined in the supernatant. The organic phase was reextracted by adding 3 ml to 4 ml 7.5 M NH_4OH and the radioactivity was determined by Cerenkov counting of the aqueous layer. For reaction times between 0.2 and 1.5 s the following experimental procedure was employed: 0.3 ml of the enzyme was added to the same volume of the reaction medium containing the ATP and Mg with continuous and vigorous stirring and at the same time an electronic stopwatch was started. The reaction was stopped with 0.3 ml of 2 M $HClO_4$ at the same time as the stopwatch.

Determination of the protein concentration

The protein concentration was determined by the method of Lowry et al. [15] using as standard bovine serum albumin, the concentration of which had been determined spectrophotometrically (A_{279} = 6.67 cm^{-1} for 1% solution

[16]). The molar concentration of the enzyme is expressed in terms of active sites assuming a molecular weight of 319 000 [17] and two catalytic sites per enzyme molecule [6—8].

Kinetic study of the time course of the reaction

The kinetics of the reaction were studied with a Durrum stopped-flow apparatus. The photomultiplier output signal was stored via a log-converter in a Datalab 905 transient recorder as a 1024 points data file, which was stored in a Hewlett-Packard 2100 A computer. The original trace was subjected to a five-points smoothing procedure, as described by Savitsky and Golay [18]. A selected part of the smoothed trace was fitted to an exponential function by means of a linear least-squares procedure, applied to the logarithm of the difference between experimental values and (estimated) final level. With the results of this linear regression method the theoretical curve could be calculated and subtracted from the original trace. This residual could be treated subsequently as described above to reveal any second exponential that might be present.

Determination of the kinetic parameters of F_1

The calculations of the turnover number (calculated per active site) and the K_m for MgATP were done by means of a computer programme essentially as described by Roberts [19]. This programme carries out a weighted linear least-squares regression analysis of the data using the Lineweaver-Burk equation. The results from the linear regression are used to determine the experimental point with the greatest deviation which is discarded. This procedure is repeated until two consecutive sets of values do not differ significantly. The kinetic parameters and the slope of the Lineweaver-Burk plot are calculated with their respective standard deviations.

Determination of the concentration of ATP and ADP

The ATP and ADP solutions were freshly prepared and the concentrations determined in the assay solutions by enzymatic assays according to Refs. 20 and 21 for ATP and ADP, respectively. The concentration of ADP in the stock ATP solution was always less than 0.5% of the total nucleotide concentration.

Results

Pre-steady-state kinetics

As pointed out by Gutfreund [22], an initial acceleration of the formation of product must occur during the first milliseconds after mixing enzyme and substrate, and the determination of the lag time (τ), defined as the intercept on the time axis of the extrapolation of the steady state, and its dependence on the substrate concentration can, in general, give some information on the rate-limiting step in the formation of the active enzyme-substrate complex. The time course of the hydrolysis of ATP measured after mixing F_1 with ATP and Mg^{2+}, shown in Fig. 1, follows qualitatively the predicted pattern. At low concentrations of MgATP (<0.1 mM), τ^{-1} is a linear function of the substrate concentration (Fig. 2), extrapolating to $\tau^{-1} = 8\ s^{-1}$ at zero substrate. This

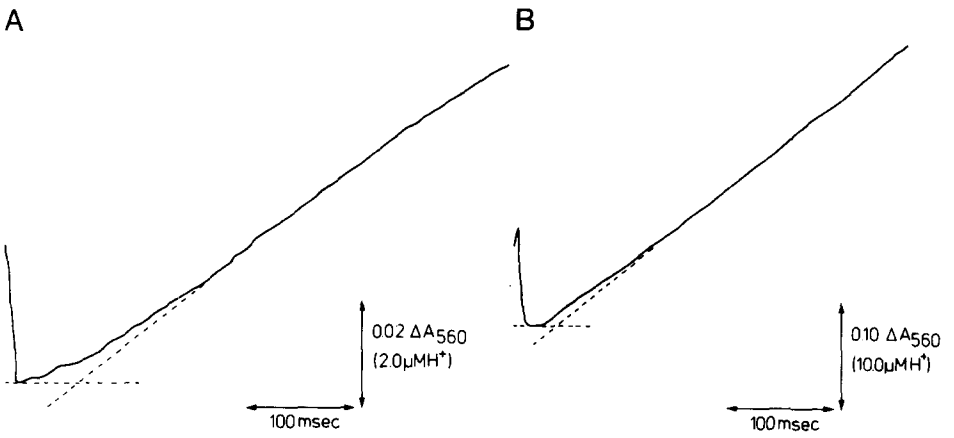

Fig. 1. Pre-steady-state kinetics of ATPase activity of F_1. The F_1 solution was dialysed against 0.25 M sucrose, 2 mM Tris-H_2SO_4 buffer and 1 mM EDTA, pH 8.0. Both syringes of the stopped-flow apparatus contained 0.25 M sucrose, 20 mM Na_2SO_4 and 0.02 mM phenol red. One syringe contained in addition 6.6 μM F_1, 50 μM EDTA and 0.2 mM Tris-H_2SO_4 buffer (pH 8.0), and the other syringe 2 mM $MgCl_2$ and 0.04 mM (A) or 0.4 mM (B) ATP. The pH values of both solutions were adjusted to 8.0 as described in Methods. The two syringes delivered equal volumes, so that the final concentrations of F_1 and MgATP, after mixing, are half those given above. The temperature was kept at 20°C. The distance on the time scale between the time of mixing and the intersection of the dotted lines gives the lag time τ.

value of τ^{-1} is much smaller than to be expected from a simple Michaelis-Menten mechanism, which predicts $\tau_{s=0}^{-1} > k_2$ (turnover number) [22] which is about 180 s^{-1} per catalytic site for F_1. In other words, the lag is much greater than predicted by Michaelis-Menten kinetics. Also in contrast to such a prediction the relationship between τ^{-1} and substrate concentration departs greatly from a straight line. Indeed, Fig. 3 shows that the relation between τ and sub-

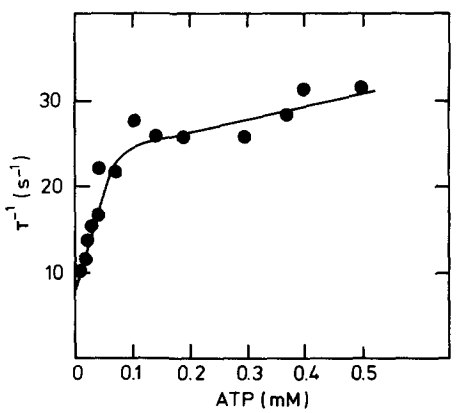

Fig. 2. Dependence of the time lag (τ) on the MgATP concentration, at low concentration of MgATP. The experimental conditions were the same as in Fig. 1. The concentration of MgATP was calculated according to Rosing and Slater [35]. The points shown are the means of at least three determinations carried out at a single concentration of MgATP with the same concentration of F_1. The different points refer to different experiments with F_1 concentrations (final after mixing) varying between 1.65 and 3.3 μM.

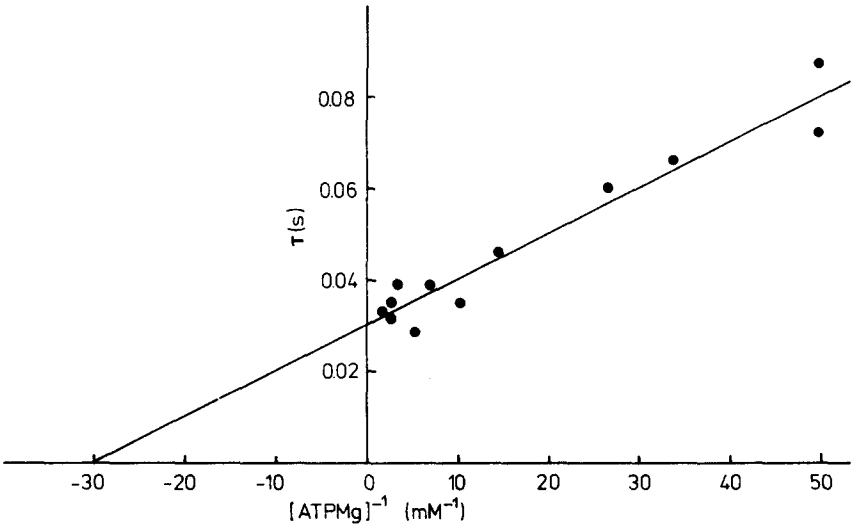

Fig. 3. Dependence of the time lag (τ) on MgATP concentration over a wider range of MgATP concentration than in Fig. 2. Otherwise, the conditions were the same.

strate concentration is hyperbolic, the half maximal being found with 30 μM MgATP. At infinite MgATP concentration τ is equal to about 30 ms.

Results similar to those shown in Fig. 1 were obtained when ATP splitting was followed by the formation of inorganic phosphate measured by a rapid-mixing and quenching procedure [23].

Steady-state kinetics

The time course of the reaction was followed at relatively high enzyme (about 3 μM) and low ATP (0.03—0.1 mM) concentrations. After the lag phase the reaction follows first-order kinetics for 1—2 s (Fig. 4) which is to be expected if the enzyme follows Michaelis-Menten kinetics with negligible product inhibition. The integrated form of the Michaelis-Menten equation for an enzyme that catalyses an irreversible reaction in which the product is a competitive inhibitor is shown in Eqn. 1

$$Vt = K_m \ln \frac{S_0}{S_0 - P} + P + \frac{K_m}{K_i}\left(S_0 \ln \frac{S_0}{S_0 - P} - P\right) \quad (1)$$

where V is the maximum velocity, S_0 the initial substrate concentration and P the product concentration. This equation reduces to a single exponential when the substrate concentration is smaller than the K_m, and the latter is smaller than the K_i for the product, or at all substrate concentrations when K_m is equal to K_i. But a single exponential would not be found if K_i were only one tenth of the K_m, as has been reported for F_1 by several authors [24,25,9]. The slight departure from first-order kinetics shortly after the end of the lag in Fig. 4B is to be expected since the ATP concentration (0.1 mM) was not much smaller than the K_m (0.24 mM).

Since the low values for K_i (ADP) reported in the literature have been

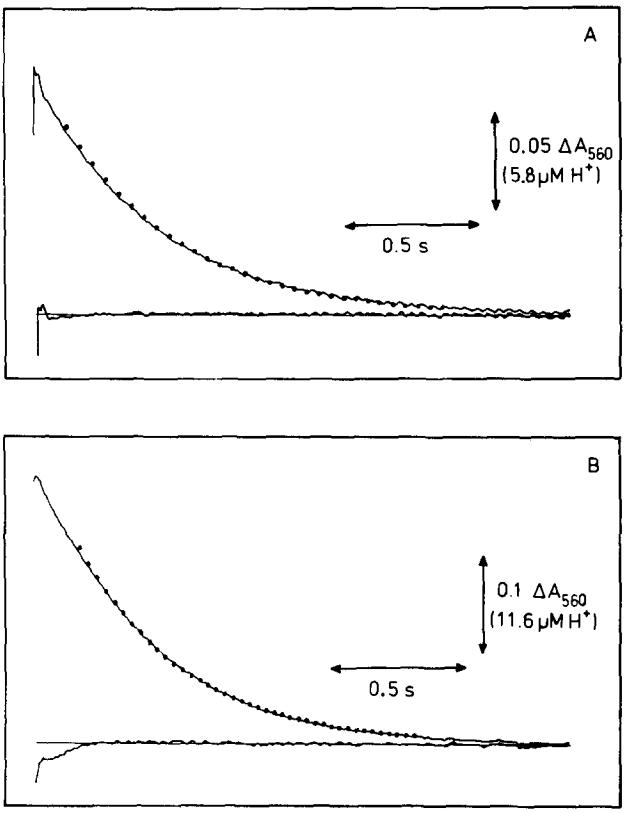

Fig. 4. Resolution of the time course of the ATPase reaction at low concentration into a single exponential. The F_1 solution used in this experiment was dialysed against 0.25 M sucrose, 2 mM Tris-HCl buffer and 20 mM NaCl. Both syringes contained 0.25 M sucrose, 20 mM NaCl, 0.3 mM Tris-HCl buffer and 0.03 mM phenol red. One syringe contained in addition 5.7 μM F_1, the other 4 mM $MgCl_2$ and 0.03 mM (A) or 0.1 mM (B) MgATP. The line represents the experimental trace and the points the theoretical value calculated for the exponential that gives the best fit (see Methods). The straight line in the lower part of each figure shows the value of ΔA_{560} at infinite time, as used in the simulation, and the curve shows the difference between the experimental and theoretical curves.

obtained at lower enzyme concentrations than used in the experiment shown in Fig. 4, the time course of the reaction was studied at different protein concentrations. To facilitate comparison, the time scales in Fig. 5 have been adjusted proportionally to the protein concentration. According to the integrated Michaelis-Menten equation, curves plotted in this way should be superimposable if the only variable in the second term of Eqn. 1 is the product concentration. In Fig. 5, it is shown that this is not the case, the kinetics deviating from first order at low protein concentration. Since no inactivation was observed when the enzyme was preincubated under similar conditions for periods longer than the longest reaction times shown in Fig. 5, it may be concluded that the deviation at low concentration is not due to denaturation of the enzyme. The time course of the reaction at the low protein concentration is compatible with a value for K_i (ADP) similar to that found by Philo and Selwyn (Fig. 1 of Ref. 25). Since the initial velocity of the reaction was found

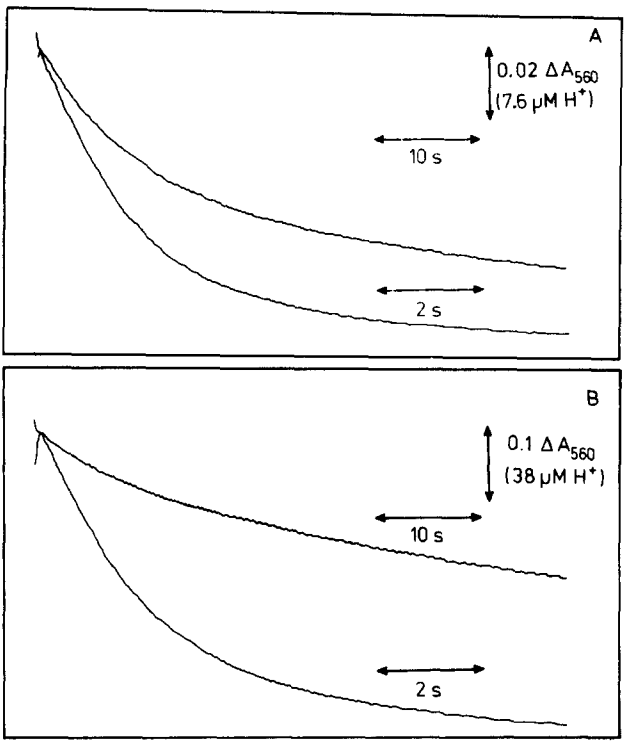

Fig. 5. Time course of the ATPase reaction over a longer period, measured at different F_1 concentrations. Conditions are as in Fig. 4, except that 1 mM Tris-HCl buffer was used, and the final concentrations of F_1 were 0.55 μM (lower trace in each figure) or 0.11 μM (upper traces). In A, 0.03 mM MgATP and in B 0.2 mM MgATP were used. The time scale has been changed in proportion to the protein concentration in order to be able directly to compare the two time courses.

to be proportional to the protein concentration both in the presence and absence of ADP (not shown), the possibility that the kinetic constants are dependent on protein concentration is excluded.

Fig. 6 shows the effect of varying both the ATP and ADP concentrations on the 'initial velocity'. The Lineweaver-Burk plots were constructed with a curve-fitting procedure as described in Methods. As is to be expected with a competitive inhibitor, ADP has no effect on the maximum velocity and its effect on the apparent K_m fits a K_i equal to 149 ± 17 μM (Figs. 6B and 6C).

When F_1 is preincubated in the reaction mixture for 2 min before starting the reaction with ATP, ADP and $MgCl_2$ in the appropriate concentrations, the trace (A in Fig. 7) follows the same course as under the corresponding conditions illustrated in Fig. 4. When, however, ADP was present during the preincubation in the same concentration as the final concentration in this experiment, and ATP, ADP and $MgCl_2$ were added in such amounts that the final concentrations of these components were the same as in the previous experiment, a very long lag (2 s) was apparent before the hydrolysis of ATP reached its maximum rate. Moreover, this maximum rate was less than half that reached in the other experiment, corresponding to a K_i for ADP of much less than 150 μM.

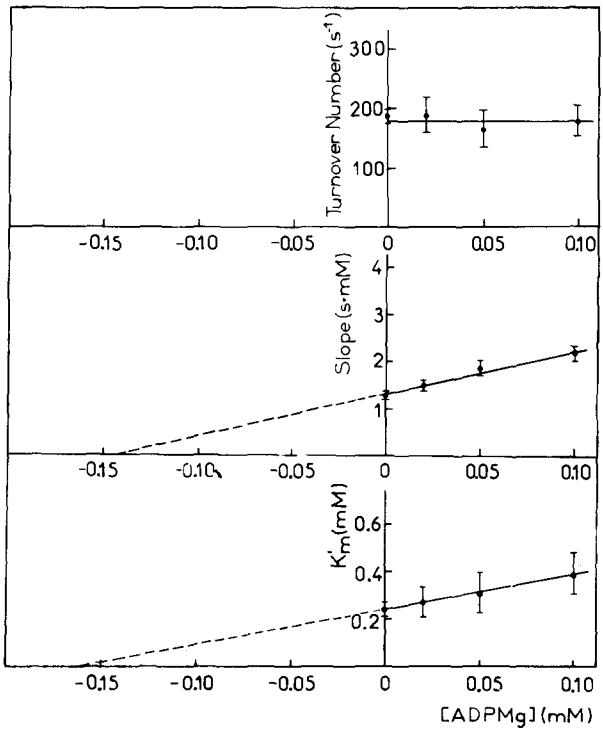

Fig. 6. Determination of the initial K_i for MgADP. The velocity of the ATPase reaction immediately after the lag phase was determined with different concentrations of MgADP and MgATP. Experimental conditions were the same as in Fig. 4, except that 2.2—2.85 μM F_1 (final concentration) was used. The parameters plotted were calculated as described in Methods. The continuous lines were calculated using a weighted linear least-squares method.

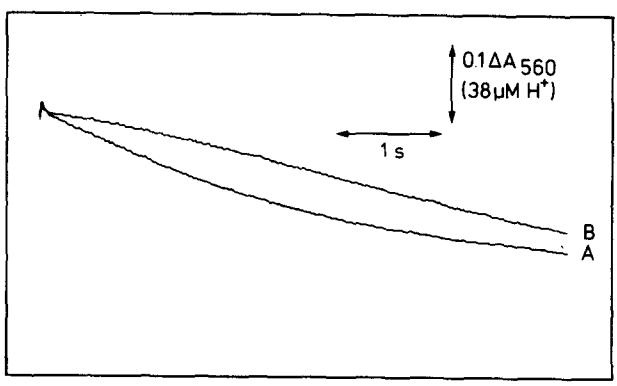

Fig. 7. Effect of preincubation of F_1 with MgADP. A. F_1 (2.2 μM) was preincubated for 2 min in a reaction medium containing 0.25 M sucrose, 20 mM Tris-HCl buffer, 0.03 mM phenol red and 0.2 mM $MgCl_2$. The reaction was then started by mixing in the stopped-flow apparatus with the same sucrose-Tris-phenol red medium containing 0.4 mM MgATP, 0.12 mM MgADP and 4 mM $MgCl_2$. B. In this experiment, F_1 was preincubated under the same conditions but 0.06 mM MgADP was also present, and the reaction was started by mixing with 0.4 mM MgATP, 0.06 mM MgADP and 4 mM $MgCl_2$, so that the final concentrations were the same in the two traces.

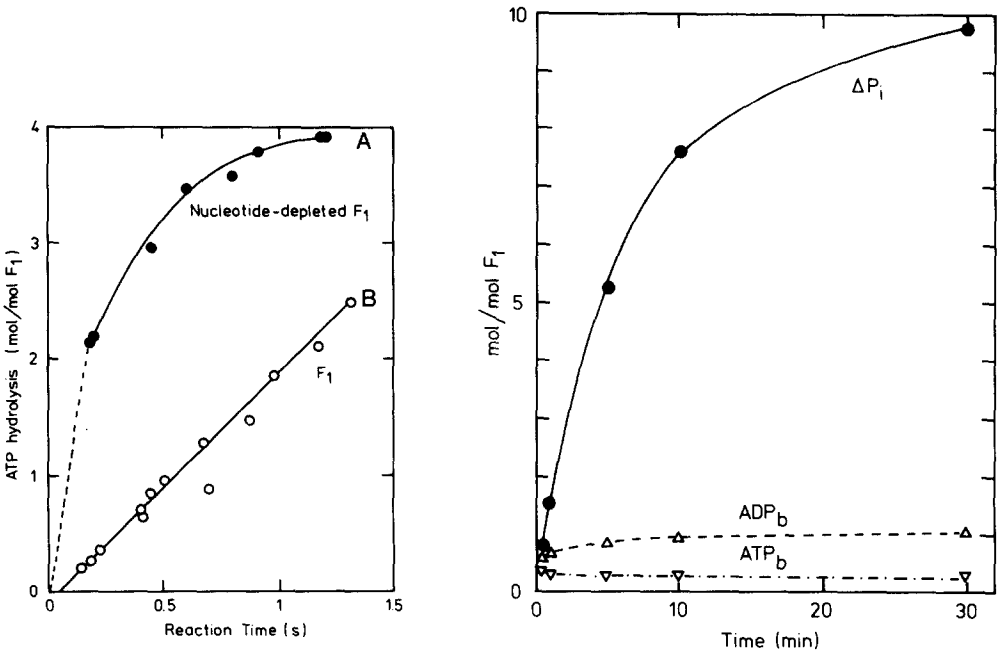

Fig. 8. Time course of hydrolysis of [γ-^{32}P]ATP catalysed by nucleotide-depleted (A) and normal (B) F_1. The reaction medium contained 50 mM Tris-H_2SO_4 buffer, 8.6% glycerol, 18.5 μM [γ-^{32}P]ATP, 1.14 mM (A) or 1.26 mM (B) $MgCl_2$ and, in B only, 0.09 mM EDTA. The reaction was started by the addition of 2 μM F_1 and stopped at the times indicated by addition of 0.5 vol. 2 M $HClO_4$. After centrifugation the amount of $^{32}P_i$ was determined in the supernatant. The nucleotide-depleted F_1 preparation contained 0.05 mol tightly bound ATP and 0.17 mol tightly bound ATP per mol.

Fig. 9. Appearance of 'tightly bound' nucleotide during hydrolysis of ATP catalysed by nucleotide-depleted F_1. Nucleotide-depleted F_1 (5.1 μM containing 0.6 mol tightly bound ATP (ATP_b) and 0.15 mol tightly bound ADP per mol) was incubated in a medium containing 98 μM [^{3}H, γ-^{32}P]ATP, 1 mM $MgCl_2$, 4 mM EDTA, 10% glycerol and 50 mM Tris-H_2SO_4 buffer (pH 8.0). The reaction was stopped at the times indicated by the addition of $(NH_4)_2SO_4$ to 50% satn. and, after centrifugation, the supernatant was acidified by adding an equal volume of 2 M $HClO_4$, and the $^{32}P_i$ determined. The pellet was washed 3 times with satd. $(NH_4)_2SO_4$ and then taken up in 0.5 ml of a solution containing 0.25 M sucrose, 10 mM Tris-H_2SO_4 buffer (pH 7.5) and 2 mM EDTA, and the protein determined. A neutralized $HClO_4$ extract was then prepared (see Ref. 13) and ^{32}P and ^3H measured in order to determine firmly bound ATP and (ATP + ADP), respectively. The radioactively labelled ATP was preincubated with phospho*enol*-pyruvate, pyruvate kinase and $MgCl_2$ for 20 min in order to convert any ADP present into ATP and the reaction stopped with excess EDTA in order to remove free Mg^{2+}.

When F_1 is depleted by glycerol treatment [12] of practically all the firmly bound ADP and ATP, the initial rate of hydrolysis of ATP is greatly enhanced (Fig. 8). Glycerol treatment had little effect on the turnover number or K_m when measured under steady-state conditions in the normal ATPase assay. In the absence of Mg^{2+}, the ADP that is slowly formed becomes bound to the glycerol-treated F_1 but little ATP is rebound (Fig. 9). This suggests that the sites responsible for tight binding of ATP (Type-I sites [5]) are modified in some way by glycerol treatment. That firmly bound ADP cannot be replaced by ATP in the absence of Mg^{2+} is consistent with the finding of ADP bound to Type-II sites in isolated F_1 kept in the presence of ATP. However, it is clearly dangerous to extrapolate the results obtained with glycerol-treated F_1 to intact F_1.

Discussion

Pre-steady state kinetics

Since the initial lag in the hydrolysis of ATP is much greater than expected from the turnover rate in the steady state, it must be concluded that the enzyme as isolated is in an inactive conformation, that MgATP in the presence of Mg^{2+} induces a transformation from an inactive to an active conformation, and that the relaxation to an inactive form is so slow that the enzyme remains in the active state during steady-state hydrolysis. Recktenwald and Hess [26] and Carmeli et al. [27] have drawn the same conclusion for yeast and chloroplast F_1, respectively. In apparent disagreement with Recktenwald and Hess [26], we find that the lag is dependent on the MgATP concentration (Fig. 3), with half-maximum activity at 30 μM ATP. This apparent disagreement can possibly be explained by the higher magnesium concentration used by the latter authors (5 mM compared with 2 mM), since under these conditions we find a lag close to 100 ms that is largely independent of MgATP concentration between 20 and 200 μM.

Since initial binding of MgATP to an empty binding site on F_1 would be expected to be very rapid (diffusion controlled), the slow relaxation from the enzymically active to the inactive conformation indicates that at least two reactions are involved in the activation. Although conclusions from the glycerol-treated enzyme must be drawn with caution, the rapid initial activity with this preparation suggests the possibility that the activation by MgATP of normal F_1 is brought about by replacement of the firmly bound ADP (to Type-II sites [5]) by MgATP. If this is the case the activation reaction could be written

$$E \cdot ADP \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ADP + E$$

$$E + MgATP \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E \cdot MgATP \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \overset{*}{E} \cdot MgATP$$

where $\overset{*}{E}$ represents the catalytically active and E the catalytically inactive conformation of the enzyme.

According to this scheme, V/e of the activation reaction

$$= \frac{k_1 k_3}{k_1 + k_3} = \frac{1}{\tau} = 33\ s^{-1}.$$

The dissociation constant of ADP bound to Type-II sites is, according to Wielders et al. [28], equal to 0.5 μM, in the absence of Mg^{2+}. If it is assumed that Mg^{2+} has little effect on this dissociation constant, we may write $k_1/k_{-1} = 5 \cdot 10^{-7}$ M and if we may assume (cf. [2]) that the binding reaction (k_{-1}) is diffusion controlled ($10^8\ M^{-1} \cdot s^{-1}$), then $k_1 = 50\ s^{-1}$, which is close enough to V/e of the activation reaction to suggest that, if this model is correct, the slow step in the activation reaction is the dissociation of ADP. It is possible, however, as suggested by Tondre and Hammes [29], that the binding of ADP is also a two-step reaction, in which case the rate-limiting step could be in the isomerization reaction. The rate-limiting rate constant in the relaxation of active to inactive conformation would presumably be k_{-3}.

The existence of this lag and also of slowly developing inhibitory reactions, combined with the fact that the turnover number of F_1 in the steady state is three orders of magnitude higher than that of myosin ATPase [2], makes it very difficult to study the kinetics of the formation of active intermediates of the ATPase reaction.

Steady-state kinetics

Taking into account competitive inhibition by MgADP, the minimum reaction sequence for the ATPase reaction may be written

$$T + E \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} T \cdot E \tag{1}$$

$$T \cdot E + H_2O \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} D \cdot E \cdot P_i \tag{2}$$

$$D \cdot E \cdot P_i \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} D \cdot E + P_i \tag{3}$$

$$D \cdot E \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} D + E \tag{4}$$

where T and D represent MgATP and MgADP, respectively.

The equilibrium constant of the first reaction ($K_1 = k_1/k_{-1}$) is equal to

$$\left[\frac{k_2}{1+\frac{k_{-2}}{k_3}}\left(\frac{K_m}{V}-\frac{1}{k_1}\right)\right]^{-1}.$$

This can be simplified to

$$\left[k_2\left(\frac{K_m}{V}-\frac{1}{k_1}\right)\right]^{-1},$$

if k_{-2} (the rate constant of the esterification reaction) is small compared with k_3 (dissociation of phosphate). This is very likely the case, since the dissociation constant of phosphate is of the order of mM, and k_{-3}, a binding reaction, is probably diffusion-controlled. This yields a value of k_3 of the order of 10^4—10^5 s^{-1}. K_m/V, calculated from the half-decay time of the exponential given in Fig. 1, is equal to $1.3 \cdot 10^{-6}$ M · s. Since k_1 is the reaction constant of a diffusion-controlled reaction, $1/k_1$ (10^{-7}—10^{-8} M · s) may be neglected. Putting $k_2 \geqslant 182\ s^{-1}$ (the turnover number of the enzyme), K_1 becomes $\leqslant 4.1 \cdot 10^3\ M^{-1}$. Making use of Penefsky's [30] data for the binding of phosphate, that given in this paper on the competitive inhibition by ADP, and the free energy of the overall hydrolysis reaction, the $-\Delta G_0$ values for partial reactions may be calculated. These are assembled in Table I and compared with data for myosin.

The agreement between the two enzymes is, at first sight, quite striking. However, data are lacking concerning the most important point relating to the mechanism of hydrolysis, and of the reverse reaction in oxidative phosphorylation, namely to which of the partial reactions of which Reaction 2 is the sum should be ascribed the large value of $-\Delta G_0$. In the case of myosin, this

TABLE I

ENERGETICS OF F_1 ATPase AT pH 8.0 AND 20°C

		ΔG°			
		F_1 [a]		Myosin [b]	
		kJ/mol	kcal/mol	kJ/mol	kcal/mol
(1) T + E	⇌ T · E	⩾−20.2	⩾−4.8	−20.5	−4.9
(2) T · E + H_2O	⇌ D · E · P	⩽−54.2	⩽−12.9	−58.6	−13.9
(3) D · E · P	⇌ D · E + P	20.2	4.8 *	12.2	2.9
(4) D · E	⇌ D + E	21.3	5.1	34.0	8.1
T + H_2O	⇌ D + P	−33	−7.8	−33	−7.8

[a] Calculated from Fig. 5 of Ref. 30 assuming no effect of ADP.
[b] From Ref. 2.

is an isomerisation of the ATP-myosin complex and not the hydrolysis reaction [2].

		ΔG° (kcal/mol)
$T \cdot M$	$\rightleftharpoons T \cdot \overset{*}{M}$	−11.0
$T \cdot \overset{*}{M} + H_2O$	$\rightleftharpoons D \cdot \overset{**}{M} \cdot P$	−1.3
$D \cdot \overset{**}{M} \cdot P$	$\rightleftharpoons D \cdot \overset{*}{M} \cdot P$	−1.6

where M, $\overset{*}{M}$ and $\overset{**}{M}$ represent identifiable conformations of myosin.

Post steady-state kinetics

Provided that the ATP concentration is sufficiently below the K_m (less than one tenth) and the concentration of the enzyme is sufficiently high so that the reaction is virtually finished within about 2 s, the hydrolysis follows first-order kinetics with a half decay time corresponding to a value of $V/K_m \cdot e$ equal to $7.7 \cdot 10^5\ M^{-1} \cdot s^{-1}$. This is in good agreement with the value ($7.5 \cdot 10^5\ M^{-1} \cdot s^{-1}$) calculated from independently determined values of the K_m (0.243 mM) and the turnover number ($182\ s^{-1}$ per catalytic site). These first-order kinetics are not compatible with a K_i for product inhibition of the order of 15—30 μM, which has been reported in the literature [9,24,25]. Indeed, the K_i calculated from rates measured immediately after the lag period, with different concentrations of ADP, was found to be about 150 μM. Since this value is consistent with the first-order kinetics, which persists for about 60 turnovers in the experiment shown in Fig. 5A (lower trace), we consider that it represents the dissociation constant of the enzyme-ADP complex in the catalytic mechanism. The lower K_i obtained after a much longer period (e.g. 300 turnovers in the experiment shown in Fig. 5A (upper trace)), must represent a slowly developing inhibitory side reaction. In principle, this could be due to either conversion of the enzyme-ADP complex that is an intermediate in the enzymic mechanism into a more tightly bound complex, or to slow binding of ADP, formed by the enzymic reaction, to a second site, resulting in inhibition of the enzymic activity. The competitive inhibition found even after pre-incubation can be

explained by competition between ADP and ATP for this second site.

Judging by the slow reactivation by ATP shown in Fig. 7, ADP dissociates very slowly from this second site.

The time-dependent inhibition of the ATPase has previously been reported by Mitchell and Moyle [31] with submitochondrial particles and by Harris et al. [32] with F_1. The latter authors showed that preincubation of F_1 with ADP causes partial inhibition of the ATPase activity and that this inhibition is relieved by pre-incubation with ATP in the absence of Mg^{2+}. They proposed that the inhibition is caused by the replacement by ADP of the two ATP molecules found in F_1 tightly bound to what Slater et al. [5] term Type-I sites. It is difficult to compare our experiments which were carried out in the presence of Mg^{2+} with those carried out by Harris et al. [32] in the absence of Mg^{2+} and at a lower pH. If the slowly developing inhibition by ADP during the course of ATP hydrolysis is due to replacement by ADP of ATP bound to Type-I sites, the affinity of ADP relative to that of ATP for this site must be much higher in the presence of Mg^{2+} than in its absence, since according to the experiments of Harris et al. [32], ATP has a much higher affinity than ADP.

In any case, our experiments support the suggestions made by Hockel et al. [33] for Micrococcus membrane F_1 and by Cantley and Hammes [34] for chloroplast F_1 that ADP bound to high-affinity sites different from the catalytic site inhibits the ATPase activity at low ATP concentration.

Acknowledgements

This work was carried out while one of us (O.A.R.) was a recipient of a post-doctoral fellowship from the Consejo Nacional de Investigaciones Cientificas y Técnicas de la República Argentina. It was supported in part by grants from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.) under the auspices of the Netherlands Foundation for Chemical Research (S.O.N.).

References

1 Pullman, M.E., Penefsky, H.S., Datta, A. and Racker, E. (1960) J. Biol. Chem. 235, 3322—3329
2 Trentham, D.R., Eccleston, J.F. and Bagshaw, C.R. (1976) Q. Rev. Biophys. 9, 217—281
3 Boyer, P.D. (1977) Ann. Rev. Biochem. 46, 957—966
4 Slater, E.C. (1977) Ann. Rev. Biochem. 46, 1015—1026
5 Slater, E.C., Kemp, A., Van der Kraan, I., Muller, J.L.M., Roveri, O.A., Verschoor, G.J., Wagenvoord, R.J. and Wielders, J.P.M. (1979) FEBS Lett. 103, 7—11
6 Wagenvoord, R.J., Van der Kraan, I. and Kemp, A. (1977) Biochim. Biophys. Acta 460, 17—24
7 Wagenvoord, R.J., Van der Kraan, I. and Kemp, A. (1979) Biochim. Biophys. Acta 548, 85—95
8 Verschoor, G.J., Van der Sluis, P.R. and Slater, E.C. (1977) Biochim. Biophys. Acta 462, 438—449
9 Schuster, S.M., Gertschen, R.J. and Lardy, H.A. (1976) J. Biol. Chem. 251, 6705—6710
10 Recktenwald, D. and Hess, B. (1977) FEBS Lett. 76, 25—28
11 Knowles, A.F. and Penefsky, H.S. (1972) J. Biol. Chem. 247, 6617—6623
12 Garrett, N.E. and Penefsky, H.S. (1975) J. Biol. Chem. 250, 6640—6647
13 Harris, D.A., Rosing, J., Van de Stadt, R.J. and Slater, E.C. (1973) Biochim. Biophys. Acta 314, 149—153
14 Bagshaw, C.R. and Trentham, D.R. (1974) Biochem. J. 141, 331—349
15 Lowry, O.H., Rosebrough, A.L., Farr, L.H. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
16 Foster, J.F. and Sterman, M.D. (1956) J. Am. Chem. Soc. 78, 3656—3660
17 Muller, J.L.M., Rosing, J. and Slater, E.C. (1977) Biochim. Biophys. Acta 462, 422—437
18 Savitsky, A. and Golay, M.J.E. (1964) Anal. Chem. 36, 1627—1639

19 Roberts, D.V. (1977) Enzyme Kinetics, pp. 285—306, Cambridge University Press, London
20 Lamprecht, D. and Trautschold, I. (1974) in Methods of Enzymatic Analysis (Bergmeijer, H.-U. ed.), Vol. 4, pp. 2101—2110, 2nd Engl. edn., Academic Press, New York, NY
21 Jaworek, D., Gruber, H. and Bergmeyer, H.-U. (1974) in Methods in Enzymic Analysis (Bergmeyer, H.-U., ed.), Vol. 4, pp. 2127—2129, 2nd Engl. edn., Academic Press, New York, NY
22 Gutfreund, H. (1975) Prog. Biophys. Mol. Biol. 29, 161—195
23 Smith, D.J., Stokes, B.O. and Boyer, P.D. (1972) J. Biol. Chem. 247, 6617—6623
24 Hammes, G.G. and Hilborn, D.A. (1971) Biochim. Biophys. Acta 233, 580—590
25 Philo, R.D. and Selwyn, M.J. (1973) Biochem. J. 135, 525—530
26 Recktenwald, D. and Hess, B. (1977) FEBS Lett. 80, 187—189
27 Carmeli, C., Lifshitz, Y. and Gutman, M. (1978) FEBS Lett. 89, 211—214
28 Wielders, J.P.M., Slater, E.C. and Muller, J.L.M. (1980) Biochim. Biophys. Acta 589, 231—240
29 Tondre, C. and Hammes, G.G. (1973) Biochim. Biophys. Acta 314, 245—249
30 Kasahara, M. and Penefsky, H.S. (1978) J. Biol. Chem. 253, 4180—4187
31 Mitchell, P. and Moyle, J. (1970) FEBS Lett. 9, 305—308
32 Harris, D.A., Gomez-Fernandez, J.C., Klungsøyr, L. and Radda, G.K. (1978) Biochim. Biophys. Acta 504, 364—383
33 Hockel, M., Hulla, F.W., Risi, S. and Dose, K. (1978) J. Biol. Chem. 253, 4294—4296
34 Cantley, L.C. and Hammes, G.G. (1975) Biochemistry 14, 2969—2975
35 Rosing, J. and Slater, E.C. (1972) Biochim. Biophys. Acta 267, 275—290