Bi-site activation occurs with the native and nucleotide-depleted mitochondrial F_1 -ATPase

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Experiments are reported on the uni-site catalysis and the transition from uni-site to multi-site catalysis with bovine heart mitochondrial F_1 -ATPase. The very slow uni-site ATP hydrolysis is shown to occur without tightly bound nucleotides present and with or without P_i in the buffer. Measurements of the transition to higher rates and the amount of bound ATP committed to hydrolysis as the ATP concentration is increased at different fixed enzyme concentrations give evidence that the filling of a second site can initiate near maximal turnover rates. They provide rate constant information, and show that an apparent K_m for a second site of about $2 \mu\text{M}$ and V_{max} of 10 s^{-1} , as suggested by others, is not operative. Careful initial velocity measurements

also eliminate other suggested K_m values and are consistent with bi-site activation to near maximal hydrolysis rates, with a K_m of about $130 \mu\text{M}$ and V_{max} of about 700 s^{-1} . However, the results do not eliminate the possibility of additional 'hidden' K_m values with similar $V_{\text{max}}:K_m$ ratios. Recent data on competition between TNP-ATP and ATP revealed a third catalytic site for ATP in the millimolar concentration range. This result, and those reported in the present paper, allow the conclusion that the mitochondrial F_1 -ATPase can attain near maximal activity in bi-site catalysis. Our data also add to the evidence that a recent claim, that the mitochondrial F_1 -ATPase does not show catalytic site cooperativity, is invalid.

INTRODUCTION

The ATP synthase and the separated F_1 -ATPase are characterized by a binding change mechanism in which catalysis at one of the three catalytic sites is strongly promoted by additional substrate binding [1–3]. However, uncertainty remains as to whether near maximal rates of ATP synthesis or hydrolysis are attained when substrates bind at a second or at a second and third catalytic site. For example, a three-site model has been proposed for ATP hydrolysis by EcF_1 (soluble ATPase portion of *Escherichia coli* ATP synthase) [4], CF_1 (soluble ATPase portion of chloroplast ATP synthase) [5] and MF_1 (soluble ATPase portion of mitochondrial ATP synthase) [6], although a two-site model for MF_1 is preferred by others [7]. Filling of two sites on ATP synthase from chloroplasts appears to suffice for near maximal rates of ATP formation [8] but, with the mitochondrial synthase, filling of three sites has been suggested as necessary for high synthesis rates [9]. The distinctions between a two-site and a three-site model are important for better understanding of the reaction mechanism. Further appraisals thus appeared warranted. One purpose of the present paper is to present evidence that, together with earlier data, demonstrates that only two sites need to be filled for rapid ATP hydrolysis by the MF_1 . Our results also provide additional characterization of the uni-site catalysis and the transition to bi-site catalysis.

In addition, the results give additional evidence to refute a recent claim that nucleotide-depleted MF_1 (nd MF_1) in the presence of P_i does not show catalytic site cooperativity [10]. Our data show such cooperativity for nd MF_1 with or without P_i present.

EXPERIMENTAL

Chemicals

ATP, NADH and BSA (essentially fatty acid free) were from Sigma, phosphoenolpyruvate monopotassium salt was from Fluka. Salt-free, freeze-dried pyruvate kinase and lactate dehydrogenase were from Sigma. The enzymes were dissolved in 50 mM Tris/HCl buffer (pH 8.0) at 20 and 10 mg/ml, respectively. Phosphoenolpyruvate at 3 mM concentration contained no detectable amounts of ADP/ATP as judged from the lack of ATPase activity measured in the presence of pyruvate kinase and MF_1 by P_i release in the absence of the added ATP. NADH preparations, however, contained about 0.5–1.0% of ADP+ATP (on the molar basis) as contamination and, if necessary (at low added ATP), ATP concentration values were corrected for the nucleotide introduced with the regenerating system. $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was prepared from $[\text{P}^{32}]\text{P}_i$ and purified as described earlier [7] and contained 1–2% of radioactivity as P_i , or, alternatively, $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was obtained from Amersham International and contained 5–10% of radioactivity as P_i .

Enzyme preparations

MF_1 was purified from bovine heart mitochondria according to the procedure of Knowles and Penefsky [11] and was depleted of tightly-bound nucleotides as described by Garrett and Penefsky [12]. The protein fractions with A_{280}/A_{260} higher than 1.95 were collected, enzyme was precipitated at 50% saturation of ammonium sulphate and the chromatography step was repeated. Nucleotide analysis by HPLC [13] showed that nd MF_1 contained

Abbreviations used: MF_1 , soluble ATPase portion of mitochondrial ATP synthase; nd MF_1 , MF_1 depleted of tightly bound nucleotides; EcF_1 , soluble ATPase portion of *Escherichia coli* ATP synthase; CF_1 , soluble ATPase portion of chloroplast ATP synthase; Ac, acetate anion.

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less than 0.1 mol of adenine nucleotide per mol of enzyme. Protein concentration was measured by the Lowry procedure [14] using freeze-dried MF_1 -ATPase as the standard. The molecular mass of MF_1 -ATPase was taken as 371 kDa [15].

Cooperative ATP hydrolysis

The rate of ATP hydrolysis under the substrate excess conditions was measured at 25 °C in a medium containing 50 mM Tris/HCl (pH 8.0), 0.1 mM EDTA, 1.1 mM MgCl_2 , 80 mM KCl, 1 mM phosphoenolpyruvate, 0.2 mg/ml pyruvate kinase, 0.1 mg/ml lactate dehydrogenase, and 300 μM NADH at MgATP concentrations from 20 to 800 μM , and 50 μM NADH at 5 to 20 μM MgATP . Two procedures were used for the initial velocity measurements. In the first, the reaction mixture additionally contained 100 μM azide, and the initial rates were calculated by extrapolation to a zero time using a semilogarithmic plot of the activity change as described earlier [16,17]. In the second approach, the MF_1 was incubated with 2 mM PP_i prior to assay, which allowed reliable measurement of the initial velocities directly from the slope of the reaction curve. Both procedures gave identical values for the initial ATPase rates.

At ATP concentrations below 5 μM , ATPase activity was measured by $[^{32}\text{P}]\text{P}_i$ release from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. The $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ hydrolysis was assayed at 25 °C in 500 μl samples containing 50 mM Tris/HCl buffer (pH 8.0), 0.1 mM EDTA, 1.1 mM MgAc_2 (Ac, acetate ion), 0.1–5 μM radiolabelled ATP as substrate and 8–12 nM MF_1 . The reaction was started by adding MF_1 , and 100 μl aliquots were quenched by mixing with 200 μl of 1% activated charcoal suspension in a solution containing 50 mM sodium phosphate and 0.5 M HClO_4 . The amount of $[^{32}\text{P}]\text{P}_i$ produced was determined in aliquots of the supernatant after centrifugation. Four time points (5–25 s) were obtained for each ATP concentration, and the amount of $[^{32}\text{P}]\text{P}_i$ produced was plotted as a function of time. The reaction was linear for at least 15 s, and the reaction rates were calculated from the slope in the first 15 s of the reaction. Less than 12% of ATP initially present was hydrolysed during the incubation. No difference in kinetics of ATP hydrolysis was found in initial velocity experiments with the native and nucleotide-depleted MF_1 preparations.

Uni-site ATP hydrolysis

Hydrolysis of 21 nM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ by 198 nM nd MF_1 was measured at room temperature in 10 μl of medium containing 20 mM Mops/Tris (pH 8.0), 0.2 mM EDTA, 2.2 mM MgAc_2 and 0.3 mg/ml of BSA (MTEMB buffer). The reaction was started by adding nd MF_1 and stopped by adding 150 μl of 0.6 M HClO_4 containing 4 mM P_i . In the cold chase experiments, 90 μl of 2.22 mM MgATP in MTEMB buffer were added for 5 s followed by 60 μl of 1.5 M HClO_4 containing 10 mM P_i . After adding 60 μl of the charcoal suspension (50 mg/ml) in 0.1 M HCl, unhydrolysed $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was removed by centrifugation, and the amount of $[^{32}\text{P}]\text{P}_i$ in 165 μl of the supernatant was determined by Čerenkov counting. Less than 3% of the added cold chase ATP was hydrolysed during the 5 s incubation as determined from a control where nd MF_1 was added to cold chase ATP and incubated for 5 s.

ATP hydrolysis with 1 μM MF_1

Earlier a procedure had been developed to measure F_1 catalytic turnover under steady-state uni-site catalysis conditions in the presence of an ATP-regenerating system [18,19]. Under these conditions the reaction rate is limited by the rate of ADP release and nearly all the nucleotide added is bound at F_1 -ATPase catalytic

sites. To measure steady-state ATP hydrolysis by 1 μM MF_1 , we used the approach described in detail earlier [18,19]. Prior to assay, nd MF_1 was exposed to 2 mM PP_i in the presence of Mg^{2+} to prevent ATP binding to the non-catalytic sites during assay at the higher ATP concentrations and to prevent MgADP -induced inhibition [20–22]. The reaction was carried out in 200 μl volume in a 50 mM Tris/HCl buffer (pH 8.0) containing 0.1 mM EDTA, 1.1 mM MgAc_2 , 80 mM KCl, 3 mM phosphoenolpyruvate and 1.0 mg/ml pyruvate kinase. An increase in pyruvate kinase concentration did not change ATPase activity, indicating that pyruvate kinase was not a rate-limiting factor in these experiments. If ATP was omitted, no detectable ATPase activity was found in the presence of either 0.01 or 1.0 μM MF_1 . Samples were incubated for 20 min at 0.1–0.7 μM ATP, 5 min at 0.8–1.2 μM ATP, 1 min at 1.4–2.5 μM ATP and 30 s at 3–5 μM ATP. The reaction was stopped by addition of 100 μl of a solution containing 2% SDS and 10 mM EDTA, and the amount of P_i produced was measured by catalysed phosphate assay [23]. The reaction was linear with time at all concentrations of ATP used.

RESULTS

Uni-site ATP hydrolysis

Recently Milgrom and Cross [24] have demonstrated that a slow uni-site catalysis occurs with nd MF_1 in the presence of P_i . They measured $[^{32}\text{P}]\text{P}_i$ formation in a short time-period from a low concentration of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ with a considerable molar excess of enzyme present, and with or without an intervening 'cold' chase with an excess of unlabelled ATP. We repeated this experiment in the absence of P_i . The results in Figure 1 show the kinetics of 21 nM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ hydrolysis by 198 nM nd MF_1 . They demonstrate clearly an initial burst and a subsequent slower phase, in accord with the expected features of uni-site catalysis (see Scheme 1 of [24,25]). The $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ binding to the nd MF_1 catalytic sites has been completed by the first time point (3 s) as shown by the nearly complete hydrolysis induced by the cold chase ($\square$). At any time as the slow uni-site catalysis proceeds, the rapid complete hydrolysis of the remaining bound $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ results when

Figure 1 Uni-site ATP hydrolysis by nd MF_1

$[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (21 nM) hydrolysis by 198 nM nd MF_1 before ($\circ$) and after the cold chase ($\square$) was measured in duplicates as described in the Experimental section. The unbroken line is drawn according to results of the best fit of the data to eqn. (1) with A_0 , A_1 and k values of 36.9%, 45.8% and 0.021 s^{-1} , respectively. The broken line represents an average of the cold chase data.

excess unlabelled ATP is added. The results demonstrate convincingly that a slow uni-site catalysis occurs with the ndMF₁ even in the absence of P_i.

Although the key characteristics of the uni-site hydrolysis observed with the ndMF₁ in the absence of P_i are akin to those reported for the MF₁ and ndMF₁ in the presence of P_i, the characteristics of individual steps appear to differ somewhat. The kinetics of ATP hydrolysis measured by the acid quench (Figure 1, ○) is described by eqn. (1):

$$A_t = A_0 + A_1(1 - e^{-kt}) \quad (1)$$

where A_t is the amount of [γ -³²P]ATP hydrolysed by the time t , A_0 is the amount of [γ -³²P]ATP hydrolysed during the burst phase, A_1 is the amount of [γ -³²P]ATP hydrolysed during the slower phase, and the k is the apparent rate constant of the slower phase. The best fit of the data of Figure 1 (○) resulted in the values of A_0 , A_1 and k equal to 36.9 %, 45.8 % and 0.021 s⁻¹, respectively. The ratio A_0/A_1 of 0.81 is an equilibrium constant, K_2 , of the second step of the MF₁ catalytic cycle under conditions when ATP binding can occur at only one catalytic site (Scheme 1 [24,25]). Since during uni-site ATP hydrolysis by ndMF₁, dissociation of the products, ADP and P_i, occurs in a random order [7], the rate constant of uni-site catalysis can be obtained from the rate constant k and the equilibrium constant K_2 as $k(1 + 1/K_2)$ and, from Figure 1, is equal to 0.047 s⁻¹.

Effect of phosphate and lower pH

Reynafarje and Pedersen [10] emphasize that their measurements were in a reaction medium near pH 7 with P_i as the only buffer. It was thus appropriate to check if such a change in pH and buffer might possibly alter the enzyme mechanism so that the slow uni-site catalysis could no longer occur. The data of Table 1 show that the occurrence of a slow uni-site catalysis, and its many-fold acceleration when ATP is available to bind at other catalytic sites is not affected by the decrease of pH of the incubation medium from 8 to 7 (compare experiments 1 and 3) or by addition of P_i into the medium (compare experiments 1 versus 2 and 3 versus 4). Experiment 5 in Table 1 shows that uni-site features were also observed when 10 mM P_i at pH 7 was used as the only buffer, i.e. under conditions used in [10]. It is of interest that P_i decreases the amount of [γ -³²P]ATP hydrolysed in the acid quench samples (Table 1). A similar observation has been made by Bullough et al. [26], who suggested that P_i can decrease the equilibrium constant of ATPase reaction in the catalytic site under uni-site conditions. The values of K_2 obtained in the present paper in the absence of P_i and in [24] in the presence of 2 mM P_i support this suggestion.

Saturation of uni-site catalysis

To provide an additional test of whether positive catalytic cooperativity occurs between the MF₁ catalytic sites, we incubated 198 nM ndMF₁ with increasing concentrations of [γ -³²P]ATP for 5 s and measured the amount of [³²P]P_i produced. Under these conditions, if there were no cooperative interactions between the catalytic sites of ndMF₁, the fraction of [γ -³²P]ATP hydrolysed would remain constant even when the substrate concentration exceeds that of ndMF₁. In contrast, if there is positive catalytic cooperativity between the catalytic sites, the [γ -³²P]ATP fraction hydrolysed during the 5 s incubation should show an increase, from the value determined mainly by the equilibrium constant of the ATPase reaction in the ndMF₁ catalytic site under uni-site conditions (about 40 %, see Figure 1) to almost complete hydrolysis when the [γ -³²P]ATP molarity significantly exceeds that of the enzyme. The results (Figure 2)

Table 1 Effects of pH and P_i on uni-site ATP hydrolysis by ndMF₁

ndMF₁ at 15 nM was incubated with 1.2 nM [γ -³²P]ATP for 15 s in the medium indicated which additionally contained 2.2 mM MgAc₂, 0.2 mM EDTA and 0.3 mg/ml of BSA (final volume 25 μ l), and the reaction was quenched (acid quench) by adding 135 μ l of 0.67 M HClO₄ containing 4.4 mM P_i. Alternatively (cold chase) after 15 s incubation, 75 μ l of 1.33 mM MgATP prepared in the corresponding medium was added for 5 s followed by 60 μ l of 1.5 M HClO₄ containing 10 mM P_i. The amount of [γ -³²P]ATP hydrolysed was measured using charcoal as described in the Experimental section. Less than 2% of the MgATP added was hydrolysed during cold chase as determined using samples where cold chase MgATP was added before ndMF₁.

Experiment	Conditions	[γ - ³² P]ATP hydrolysed (%)	
		Acid quench	Cold chase
1	20 mM Mops/Tris (pH 8)	55.3	85.6
2	20 mM Mops/Tris (pH 8) + 10 mM P _i	24.6	78.2
3	20 mM Mops/Tris (pH 7)	54.7	86.0
4	20 mM Mops/Tris (pH 7) + 2 mM P _i	34.9	95.7
5	10 mM P _i (pH 7)	27.1	82.1

Figure 2 [γ -³²P]ATP hydrolysis by 198 nM ndMF₁

[γ -³²P]ATP hydrolysis by 198 nM ndMF₁ at nucleotide concentrations from 20 nM to 1 μ M was measured for 5 s in duplicates as described for uni-site hydrolysis in the Experimental section. The lines are the linear regression lines obtained using the linear segments of the curve.

show that initially, when the ndMF₁ concentration is significantly higher than that of [γ -³²P]ATP, the amount of [³²P]P_i formed is proportional to the amount of [γ -³²P]ATP added. This is expected since most of [γ -³²P]ATP binding takes place without formation of the enzyme species with two (or all three) catalytic sites occupied, so the [γ -³²P]ATP hydrolysis occurs mostly through uni-site mode. Then the amount of substrate hydrolysed during 5 s should be a constant fraction of the amount of substrate added, reflecting the equilibrium between bound substrate and products at the catalytic site.

When the concentration of added [γ -³²P]ATP in Figure 2 approaches that of ndMF₁, the extent of the nucleotide hydrolysis begins to increase. This increase becomes more pronounced when the concentration of [γ -³²P]ATP exceeds that of ndMF₁. In the presence of 1 μ M ATP, nucleotide binding to the non-catalytic sites of ndMF₁ occurs too slowly [21] to be significant during the time for the experiment reported in Figure 2. This

means that the nucleotide binding site whose filling results in an acceleration of the enzyme turnover is a catalytic one. The regression lines obtained for the linear parts of the curve in Figure 2 intersect at a point corresponding to about 190 nM of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. This is close to the ndMF_1 concentration used. The second of these lines intercepts the x -axis at 95 nM, close to the concentration of ATP which is expected to be in equilibrium with the products at the catalytic sites when only one catalytic site of MF_1 is saturated, $109 \text{ nM} = [\text{F}_1]/(1 + K_2)$. These facts show that the slow ATP hydrolysis, as noted in Figure 1, involves only one catalytic site of ndMF_1 . Similar results were obtained when the experiment reported in Figure 2 was repeated using 99 nM ndMF_1 , but in this case the respective intersection points were at 103 nM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, satisfactorily close to the ndMF_1 concentration used, and at 51 nM, reasonably close to the expected value of 54.7 nM (not shown). These results show that ndMF_1 has cooperative interactions similar to those reported for the preparations of MF_1 containing non-catalytic nucleotides [27,28].

Effect of ATP concentration on the amount of bound ATP committed to hydrolysis

An alternative explanation for the results of Figure 2 is that ATP binding to the second catalytic site does not produce any significant acceleration of the product dissociation from the first catalytic site, but only shifts the equilibrium of the bound reactants toward ADP and P_i , and it is substrate binding to the third catalytic site that promotes product release from the first catalytic site. Indeed, such a mechanism has been recently proposed for the *E. coli* F_1 [29]. To examine whether this might be the case with the mitochondrial F_1 , we investigated how the amount of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ bound to ndMF_1 and committed to hydrolysis would change with the amount of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ added. This bound ATP committed to hydrolysis can be measured as a difference in the amount of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ hydrolysed during short incubations with and without cold chase (Figure 3). The amount of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ committed to hydrolysis gradually increases until the concentration of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ added reaches that of ndMF_1 (153 nM). Further increases of the $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ concentration, up to 300 nM, result in little if any additional increase in the amount bound and committed to hydrolysis. With two moles of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ added per mole of ndMF_1 , the amount bound that is committed to hydrolysis is about 0.4 mol/mol ndMF_1 . This is close to a value of 0.55 mol of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ /mol ndMF_1 that is expected from the equilibrium constant (Figure 1) at complete saturation of one catalytic site.

For a mechanism such as that suggested for EcF_1 [29], the amount of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ committed to hydrolysis should not show a saturation when the concentration of added $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ is approaching that of F_1 , but continue to increase afterwards toward the value close to that of the enzyme concentration until the second catalytic site on F_1 has been saturated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. The results of Figure 3 thus support the conclusion that ATP binding to the second catalytic site promotes the product release step in the first catalytic site, and that the second catalytic site assumes the properties of the first one after the products have dissociated from the first catalytic site.

Transition from uni-site to multi-site catalysis

The data of Figures 1–3 and Table 1, and in [24], are consistent with near maximal velocity resulting when a second catalytic site binds ATP. But they do not discern adequately whether additional acceleration may occur when a third catalytic site is filled. Therefore an additional experiment, similar to the one reported in Figure 2, was conducted but with an approx. 5-fold

Figure 3 $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ committed to hydrolysis at the catalytic sites of ndMF_1

$[\gamma\text{-}^{32}\text{P}]\text{ATP}$ hydrolysis by 153 nM ndMF_1 at nucleotide concentrations from 10 to 300 nM was measured for 5 s in duplicates as described for uni-site hydrolysis in the Experimental section, and the amount of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ committed to hydrolysis was obtained as a difference in the amount of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ hydrolysed with and without cold chase.

higher enzyme concentration, a range of ATP concentration from 0.1 to 5 μM , and with measurement of ATPase activity by P_i released. Pyruvate kinase and phosphoenolpyruvate were present to regenerate ATP from ADP released during multiple F_1 turnovers, conditions similar to those used earlier [18,19]. The results are given in Figure 4, and were assessed in the following manner.

Figure 4 Rate of ATPase reaction catalysed by 1 μM MF_1 at various concentrations of added ATP

ATP hydrolysis rate was measured from P_i production as described in the Experimental section. The continuous line represents theoretical curve obtained by assuming that saturation of the second catalytic site with K_m of 129 μM results in $V_{\max}$ of 632 s^{-1} . The broken line represents the theoretical curve obtained by assuming that saturation of the second catalytic site with K_m of 2 μM results in $V_{\max}$ of 9.8 s^{-1} , and that saturation of the third catalytic site with K_m of 129 μM results in $V_{\max}$ of 632 s^{-1} . Inset shows the data at ATP concentrations $< 1 \mu\text{M}$. The unbroken line in the inset is drawn according to eqn. (2), using k_1 , k'_1 and $k_{\text{uni,obs}}$ values of 5.0×10^6 , $5.3 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ and 0.018 s^{-1} , respectively.

When low concentrations of ATP are added, nearly all the ATP is bound by the excess enzyme present [18,19] and a slow steady-state ATP hydrolysis occurs (Figure 4, inset). Under these conditions, the rate of ATPase reaction (v) is determined by eqn. (2):

$$v = e_s(k_{\text{uni,obs}} + k'_1 s_t) \quad (2)$$

where e_s is the concentration of the enzyme:substrate (enzyme:products) complex which is formed both through uni-site ATP binding and product dissociation from bi-site intermediate, s_t is the concentration of free ATP, and k'_1 is the second-order rate constant of ATP binding during bi-site catalysis; $k_{\text{uni,obs}}$ is the observed rate constant that is equal to $k_{\text{uni}}K_2/(1 + K_2)$, where k_{uni} and K_2 are the rate constant of uni-site catalysis and the equilibrium constant of ATPase reaction at the catalytic site, respectively. Since the concentration of the free ADP in the system is very low (a value of about 3 nM can be obtained when ATP hydrolysis rate is 0.1 $\mu\text{M/s}$) and the concentration of the nucleotides bound to pyruvate kinase is even lower, both can be neglected in the balance of the nucleotides. Therefore the concentration of e_s is equal to concentration of the nucleotide bound to F₁:

$$e_s = s_t - s_f \quad (3)$$

where s_t is the concentration of added ATP. During the steady state, the rate of ATP hydrolysis (v , eqn. 2) is equal to the rate of ATP binding to F₁, v_b , which is determined by the expression:

$$v_b = s_t[(e_t - e_s)k_1 + e_s k'_1] \quad (4)$$

where e_t is the concentration of F₁ added, and k_1 is the second-order rate constant of ATP binding during uni-site catalysis. From the eqns. (2–4), the concentration of free ATP, s_f , can be obtained as:

$$s_f = \frac{\{(e_t - s_t + k_{\text{uni,obs}}/k_1)/2\}^2 + s_t k_{\text{uni,obs}}/k_1\}^{1/2} - (e_t - s_t + k_{\text{uni,obs}}/k_1)/2}{1} \quad (5)$$

and substituted into eqn. (2). With the k'_1 value of $5.3 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ (obtained below from the data of Figure 5), fitting the data of inset (Figure 4) to the eqn. (2) resulted in the values of $k_{\text{uni,obs}}$ and k_1 equal to 0.018 s^{-1} and $5.0 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$, respectively.

Using the value of the equilibrium constant of the ATPase reaction in the catalytic site of MF₁, 0.81 (Figure 1), the k_{cat} value for uni-site catalysis can be obtained from the data of the inset in Figure 4 to be about 0.04 s^{-1} , that is close to the value obtained above from the single-turnover experiment (Figure 1) and earlier for MF₁ [18,19,24,30], and also to the rate of P_i dissociation during uni-site catalysis obtained recently for EcF₁ [31,32]. However, this value is higher than those reported by others for MF₁ [25,26,28] or EcF₁ [33,34]. Reasons for this difference are uncertain. They may, at least in part, reflect differences in the enzyme preparations used, e.g. different non-catalytic site occupancy [35].

When the concentration of the added ATP approaches that of the MF₁ (1 μM), the ATPase rate shows a remarkable rise (Figure 4, inset) that is owing to simultaneous increase in the concentrations of the uni-site complex, eqn. (3), and free ATP, eqn. (5). The rapid hydrolysis of ATP with the rate proportional to the concentration of free ATP begins when the added ATP concentration becomes greater than the concentration of MF₁ present, just above 1 μM (Figure 4). This results in a sharp break at 1 μM ATP. A bimolecular rate constant for ATP binding at added ATP concentrations higher than 1 μM (Figure 4) is $4.9 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ which is close to the value of $5.3 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ obtained from the initial rate measurements at low enzyme

Figure 5 Dependence of the MF₁ turnover rate on ATP concentration

Initial ATPase activity was determined as described in the Experimental section. A coupled assay was used at ATP concentrations 5–800 μM , and [³²P]P_i-release assay at ATP concentrations below 5 μM . (A) 0–1 μM ATP; (B) 0–10 μM ATP; (C) 0–100 μM ATP. The unbroken line represents a theoretical Michaelis–Menten curve calculated using K_m and V_{max} values of $133 \pm 11 \mu\text{M}$ and $700 \pm 23 \text{ s}^{-1}$, respectively, obtained from the best fit of the data. The dotted line is calculated assuming only a 10-fold increase in k_{cat} for bi-site catalysis (to 0.4 s^{-1}) with K_m of 6 μM , as suggested in [5], and that saturation of the third catalytic site with K_m of 133 μM results in V_{max} of 700 s^{-1} . The data are mean $\pm$ S.D. of 3 or 4 experiments with two MF₁ preparations.

concentration (see Figure 5 below). Thus, the break in the Figure 4 is owing to a transition from uni-site to bi-site ATP hydrolysis that occurs when ATP binds to enzyme having one occupied catalytic site. The multi-site mode of catalysis is characterized by a single set of K_m and V_{max} of about 130 μM and 700 s^{-1} , respectively (see Figure 5), which can satisfactorily describe the transition to bi-site catalysis (Figure 4).

The unbroken line in Figure 4 represents a computer simulation of the dependence of ATPase rate on the added ATP concentration obtained by assuming that the maximal MF₁ activity is reached when only two of the three catalytic sites are occupied by the nucleotides (using for bi-site catalysis the K_m and V_{max}/K_m values of 129 μM and $4.9 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$, respectively). This line fits satisfactorily the experimental data. However, the experimental data deviate significantly from the theoretical lines obtained under assumption that the maximal MF₁ activity is reached when all three catalytic sites are occupied and bi-site catalysis has K_m and V_{max} values in the ranges suggested [6,36], e.g. of 2 μM and 10 s^{-1} for the broken line of Figure 4.

Cooperative ATP hydrolysis

A number of reports have shown that when MgADP-induced inhibition of MF₁-ATPase is avoided by measuring initial reaction velocities, or by adding the activating anions, or by prior exposure of the enzyme to pyrophosphate, a double-reciprocal plot with ATP concentrations above 5–10 μM is linear [16,19,20,37–39]. However, inadequate results are available in the literature for the initial velocities in the presence of lower ATP concentrations. Some careful measurements with relatively high enzyme concentration are reported in Figure 4. In addition, we measured initial velocities of ATP hydrolysis over a wide range of ATP concentrations (0.1–800 μM) using lower enzyme concentration, and with assay conditions and precautions as described in the Experimental section to assure reliable initial velocity values. Results are reported in Figure 5.

Rates of ATP hydrolysis at the substrate concentrations higher than 5 μM were obtained using the conventional assay coupled to NADH disappearance, and an assay measuring [³²P]P_i release from [γ -³²P]ATP was used at lower substrate concentrations. At

5 μM ATP, both methods gave practically identical rates. Results with ATP concentration ranges 0–1, 0–10 and 0–100 μM are shown in the panels of Figure 5. The best fit of the data to Michaelis–Menten kinetics in the entire concentration range (unbroken line) gave K_m and $V_{\max}$ values of $133 \pm 11 \mu\text{M}$ and $700 \pm 23 \text{ s}^{-1}$, respectively. A bimolecular rate constant calculated as $V_{\max}/K_m$ is about $5.3 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$. The experimental points show no detectable deviations from Michaelis–Menten kinetics in Eadie–Hofstee, Lineweaver–Burk or Hanes plots of the data.

These results are entirely consistent with bi-site activation giving rapid catalysis in MF_1 . The data of Figure 5 disagree with the models involving only a small increase in k_{cat} upon transition from uni-site catalysis to bi-site catalysis with K_m in the micromolar range. This is illustrated by the dotted line in Figure 5 that is calculated assuming a 10-fold increase in k_{cat} for bi-site catalysis with K_m of 6 μM , as suggested by Andralojc and Harris [5] for CaATP hydrolysis by CF_1 . However, initial velocity measurements, as in Figure 5, do not suffice to eliminate the possible participation of additional catalytic sites with K_m values below about 100 μM and with ratios of $V_{\max}$ to K_m near to those that yield the unbroken line in Figure 5. Fortunately, however, as noted in the Discussion, other recent data obtained with use of TNP-ATP [7] show that MF_1 has a catalytic site that binds ATP with a K_d in the millimolar range. Thus only two catalytic sites are available to explain the data at lower ATP concentrations as reported in this paper.

DISCUSSION

Our results give a more thorough description and analysis of the transition from uni-site to multi-site catalysis by the F_1 -ATPase than presented previously. The results and those reported recently by Milgrom and Cross [24] are in full accord with the binding change mechanism [1,2] and refute the claim of Reynafarje and Pedersen [10] that the ndMF_1 does not show cooperativity between catalytic sites.

Reynafarje and Pedersen [10] may have failed to observe the slow uni-site catalysis due to inefficiency of the charcoal in preventing the hydrolysis of ndMF_1 -bound $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ without denaturing the enzyme [24]. In addition, the luciferase assay used measures the disappearance of medium ATP, not necessarily its hydrolysis. The second-order rate constants for ATP binding to MF_1 during uni-site or bi-site catalysis are nearly identical [25,27]. Thus, when enzyme is present in excess, the rapid binding could be mistakenly interpreted as rapid hydrolysis, and the slow uni-site rate would remain undetected.

Although some small differences in uni-site catalysis characteristics apparently occur with the nucleotide-depleted enzyme as compared with the enzyme as usually isolated with two non-catalytic nucleotides present [40,41], the principal features of the binding change mechanism are operative with both enzyme preparations. The only well-demonstrated function for the non-catalytic nucleotides is that their presence facilitates the overcoming of the strong Mg^{2+} - and ADP-induced inhibition of the ATPase activity [13,21,36,42]. Other facets of their function remain to be elucidated.

The experiments on the transitions from uni- to multi-site catalysis (Figures 2 to 4) and the initial velocity measurements (Figure 5) are satisfactorily explained by the MF_1 having a binding site with a K_m well below 1 μM that gives uni-site catalysis and a second site, with a K_m near 130 μM , that gives rapid bi-site catalysis. As noted, initial velocity measurements could fail to detect the presence of other binding sites in the concentration ranges covered by our experiments if the ratios of their $V_{\max}$ to K_m values were similar to those proposed for bi-site

catalysis. It is well recognized that increases in ATP concentration above 0.5–1 mM do not give any appreciable increase in the activity of MF_1 . Importantly, a recent study showed that, in the presence of ATP, TNP-ATP could enter the catalytic sequence by binding to a catalytic site with a very low affinity for ATP (about 2 mM) [7]. That this site is indeed catalytic was confirmed using a photoactive 2-azido analogue of TNP-ATP [43]. Obviously, this catalytic site remains mostly unoccupied when the bi-site ATP hydrolysis with a K_m of about 0.13 mM is approaching a maximum rate.

It seems probable to us that other F_1 -ATPases also need to fill only two catalytic sites to reach near maximal velocity, and that this may also be the case for ATP formation by the synthase. With CF_1 in chloroplasts, where it was possible by application of a rapid filtration during photophosphorylation to measure medium and bound ADP, the filling of only two sites seemed sufficient for rapid net synthesis [8]. Data of Matsuno-Yagi and Hatefi [9] are also consistent with rapid bi-site ATP synthesis if, as suggested elsewhere [2], one of their very tightly bound ADP should not be considered as a separate catalytic participant. With both the chloroplast and mitochondrial synthases more study is needed to gain a satisfactory understanding.

Comment is necessary about recent studies of Weber et al. [4,29,44,45] with the *E. coli* F_1 . They replaced tyrosine at catalytic sites with tryptophan and demonstrated that catalytic site binding could be detected by measurement of fluorescence quenching. However, from the ATP-concentration dependence of the quenching they concluded that three catalytic sites must be filled for rapid ATP hydrolysis (tri-site activation). This conclusion is based on at least two major assumptions: (i) there is only one active species of EcF_1 in solution and (ii) that all nucleotide· EcF_1 complexes detected through fluorescence quenching represent genuine intermediates of the catalytic cycle. Unfortunately, the presence of ϵ subunit in the EcF_1 preparations employed by Weber et al. [4,44,45] makes the first assumption very unlikely, not only because the dissociation of ϵ subunit during experiments would give rise to the enzyme form with much higher activity [46–48], but also because of possible partial loss of ϵ during the purification procedure. The fact that free ATP significantly inhibits MgATP hydrolysis by EcF_1 [49] and the demonstration that EcF_1 , despite an earlier claim to the contrary [50], can form an inactive complex when MgADP is bound to one of the catalytic sites [48,51] argue strongly against the validity of the second assumption. Indeed, all the inactive complexes containing nucleotides at the catalytic sites, arising either from formation of the inhibitory MgADP at one of the catalytic sites, or from the binding of free ATP instead of the true substrate MgATP , would contribute to fluorescence quenching.

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