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A kinetic study of the binding of an ADP fluorescent analog to mitochondrial ATPase

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SUMMARY

The binding of the ADP fluorescent analog 3-β-D-ribofuranosylimidazo[2,1-*i*]-purine 5′-diphosphate to a tight binding site on solubilized beef heart mitochondrial ATPase has been found to be slow relative to most enzyme–ligand interactions, suggesting a conformational change may be rate limiting in the binding process. The effect of ADP binding to the same enzyme site on the rate of enzymatic ATP hydrolysis is small, although slight activation and inhibition may occur at low and high ATP concentrations, respectively.

Mitochondrial ATPase is an important enzyme in oxidative phosphorylation[1,2]. The enzyme from beef heart, which has been solubilized and purified[1,3,4], has a very complex subunit structure[5–7], probably reflecting the complex regulatory processes and interactions with the membrane that must be associated with this enzyme. The solubilized ATPase of beef heart mitochondria has been shown to possess two sites for the binding of ADP with dissociation constants of approximately 0.3 μM and 30 μM in the presence of Mg^{2+} at pH 8 and room temperature[8]. The weakest binding site has a dissociation constant very similar to the steady-state kinetic inhibition constant for ADP[9] and therefore is very probably a catalytic site. The functional role of the tight binding site remains to be clarified.

In this note the rate constants associated with the binding of an ADP fluorescent analog, 3-β-D-ribofuranosylimidazo[2,1-*i*]purine 5′-diphosphate (ε-ADP)[10], to the tight binding site of the solubilized enzyme have been obtained, and the effect of very low concentrations of ADP on the enzymic activity was examined.

Materials. The solubilized ATPase from beef heart mitochondria was prepared as

Abbreviation: ε-ADP, 3-β-D-ribofuranosylimidazo[2,1-*i*]purine 5′-diphosphate.

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described by Horstman and Racker[1], with beef heart mitochondria generously provided by Dr E. Racker. The specific activity of the enzyme was typically 90–100 μmoles/min per mg.

The ATP which is present in the ammonium sulfate suspension of the purified enzyme[8] was removed before carrying out kinetic experiments by dissolving the amount of centrifuged ammonium sulfate precipitate necessary for each experiment in a buffer containing 0.025 M sucrose, 10 mM Tris–HCl, 2 mM EDTA, pH 8, and passing the solution through a Sephadex G-25 medium column equilibrated with the same buffer. The eluted enzyme, identified by its absorption at 280 nm, was then precipitated with an equal volume of saturated ammonium sulfate at pH 7 and kept at 4 °C for half an hour. The ATP, with a maximum absorption at 259 nm, was always well separated from the enzyme. The ammonium sulfate precipitate of ATP-free enzyme was then centrifuged at 4 °C and the precipitate dissolved in a minimal volume at pH 8 in 0.1 M NaCl, 2 mM $MgCl_2$, 50 mM Tris–HCl. Finally a small Sephadex G-25 column equilibrated with the same solvent was used to remove the ammonium sulfate without appreciable dilution of the enzyme solution. The enzyme was then used as quickly as possible because of the denaturation which occurs in the absence of ATP[11].

The concentrations of the enzyme solutions were determined using the procedure of Lowry *et al.*[12] with human serum albumin as standard and the known correction factor of 1.18 (ref. 13). The molecular weight of the enzyme prepared by the above method is assumed to be 285 000 (refs 7, 14), although a higher molecular weight has been reported for other preparations[5,6].

The ε-ADP was prepared as previously described[10,8]. The other nucleotides (ATP and ADP) were obtained from Sigma and were used without any further purification, after checking their purity by thin-layer chromatography in the solvent system 0.15 M citric acid (pH 4), 95% ethanol, *n*-butanol (6:10:1, v/v/v). All other chemicals used were of the highest available purity. Solutions were prepared from distilled deionized water.

Kinetic experiments. A Hitachi Perkin–Elmer model MPF-3 fluorescence spectrometer operating in the ratio mode with a 2- to 3-fold scale expansion was used to measure the fluorescence change at 410 nm (maximum emission for ε-ADP) accompanying the binding of ε-ADP to the enzyme. The excitation wavelength was 310 nm and the emission was filtered in order to eliminate the contributions of wavelengths under 390 nm (filter 39). The procedure used to study the kinetics of ε-ADP binding to the enzyme was as follows: a very small amount (usually 1 microliter) of a concentrated solution of ε-ADP (2 mM) in water was added to 2 ml of buffer in a fluorescence cell thermostated at 25 °C, using a Hamilton syringe with a Chaney adapter. After mixing the solution the emission slit and zero suppression were adjusted to the desired sensitivity. The identical experiment was then carried out, replacing the buffer by the enzyme solution, and the fluorescence change was measured as a function of time within 20 s of mixing.

Initial velocities were measured using the pH-stat technique[9]. Stopped-flow and temperature-jump experiments were carried out with the stopped-flow–temperature-jump apparatus described elsewhere[15,16].

A typical plot of the change in fluorescence *versus* time after mixing ATPase and

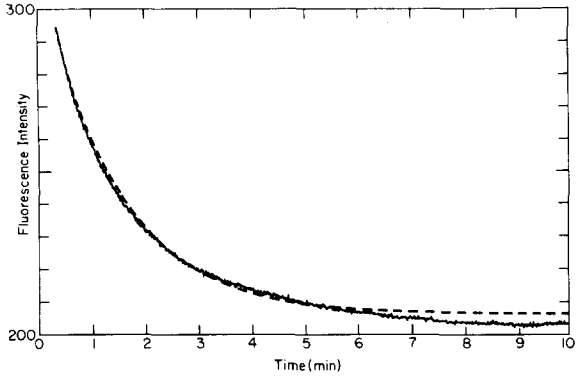

Fig. 1. The change of fluorescence at 410 nm (arbitrary units) accompanying the binding of ϵ-ADP (initial concentration 1 μM) to ATPase (initial concentration 19.7 μM) versus time at pH 8.0, 25 °C, 50 mM Tris–HCl, 0.1 M NaCl, 2 mM $MgCl_2$. The dashed line is the non-linear least squares best fit assuming the decay in fluorescence is exponential.

ϵ-ADP is shown in Fig. 1. For all of the experiments at different enzyme and ϵ-ADP concentrations, the fluorescence intensity extrapolated to zero time was approximately equal to the sum of the separate fluorescence intensities of ϵ-ADP and enzyme. The absence of a more rapid change in fluorescence was confirmed by failing to detect any fast relaxation processes with stopped-flow and temperature-jump techniques. The quenching of fluorescence, which was not observed in the absence of Mg^{2+}, could be readily reversed by ADP, which competes for the ϵ-ADP binding site. The rate progress curves could be fit quite well by assuming that the reaction was first order in ϵ-ADP and that the change in fluorescence decayed exponentially to an equilibrium value (Fig. 1). The data could not be fitted with an integrated second order rate equation. If the simple binding reaction of Eqn 1 is assumed and the enzyme concentration is always much greater than that of ϵ-ADP,

$$E + \epsilon\text{-ADP} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E - \epsilon\ ADP \tag{1}$$

the pseudo first order rate constant, k, is given by Eqn 2. In this equation $[E_0]$ is the total enzyme concentration

$$k = k_1[E_0] + k_{-1} = k_1([E_0] + K_d) \tag{2}$$

and K_d is the dissociation constant, which is about 0.5 μM[8]. A plot of the pseudo first order rate constant *versus* $([E_0] + K_d)$ is shown in Fig. 2 for experiments where the total concentration of ϵ-ADP is 1 μM and $[E_0] \geqslant 5$ μM. The values of the rate constants obtained from a least squares analysis of these data are $k_1 = 0.63 \cdot 10^3\ M^{-1} \cdot s^{-1}$ and $k_{-1} = 3.2 \cdot 10^{-4}\ s^{-1}$ (pH 8.0, 25 °C). The uncertainty in the rate constants is estimated to be ± 20%.

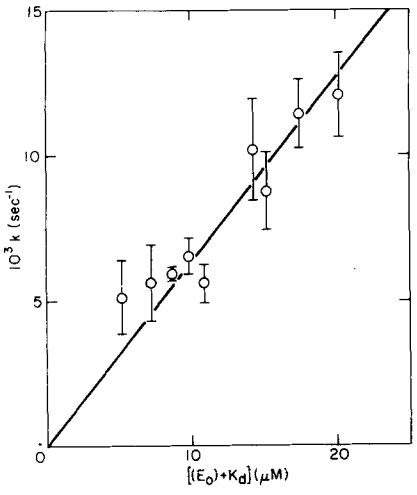

Fig. 2. A plot of the pseudo first order rate constant versus the sum of the total enzyme concentration, $[E_0]$, and the equilibrium dissociation constant, K_d, at pH 8.0, 25 °C, 50 mM Tris–HCl, 0.1 M NaCl, 2 mM $MgCl_2$, and 1 μM ε-ADP. The straight line is a linear least squares fit of these data.

The second order rate constant characterizing the binding of ε-ADP to enzyme is unusually small compared to the rate constants usually associated with enzyme–substrate complex formation[17]. A conformational change of the enzyme may be rate limiting in the binding process.

Since the binding of ε-ADP to the tight site requires several minutes for appreciable reaction to occur at the concentrations utilized, any examination of the functional role of ADP (which has been found to have a binding constant very similar to that of ε-ADP)[8] binding to the same site requires preequilibration of the enzyme and ADP. Both initial velocity and stopped-flow measurements of ATP hydrolysis were carried out with enzyme preincubated with varying amounts of ADP (1–20 μM). For the pH-stat measurements the experimental conditions were chosen so that the amount of ADP produced by the enzymatic reaction during the measurement was always less than 10 μM. The results obtained indicate that at low ATP concentrations, the binding of ADP may slightly activate the enzyme, but the magnitude of the activation, about 10%, is within the experimental uncertainties. At high ATP concentrations (approx. 1 mM), where more precise data can be obtained, the binding of ADP to the tight site is slightly inhibitory (10–20%). Thus the tight binding site for ADP on the solubilized enzyme does not possess strong allosteric regulatory properties, and its physiological function remains to be determined. The binding of ADP to the chloroplast ATPase has a much larger effect on the rate of the enzymatic reaction[18].

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