A SLOW CONFORMATION CHANGE IN THE TRANSIENT STATE KINETICS OF SOLUBLE ATPase OF YEAST MITOCHONDRIA

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1. Introduction

Soluble ATPase of mitochondria is now an accepted component of the enzyme system catalyzing oxidative phosphorylation. According to the hypothesis of Boyer and Slater [1,2] a conformational transition of the enzyme protein is expected to be involved in ATP synthesis and the kinetic resolution of the ATPase reaction should give further insight into the mechanism of energy conservation in oxidative phosphorylation. The high purity of an ATPase preparation obtained in this laboratory [3] allowed to analyze the transient state kinetics of the hydrolytic reaction catalyzed by the enzyme. We here report that the catalytic process can be resolved into two steps, one of which is a slow conformational transition with a time constant of the order of 10 sec^{-1} followed by a steady state hydrolysis with a turnover number of 200 sec^{-1}. A preliminary report of this work was given elsewhere [4].

2. Material and methods

The soluble ATPase was prepared according to [3]. The specific activity of the enzyme was about 150 U/mg. The turnover number is given under the assumption of three active sites on the enzyme. The overall activity was determined in an ATP regenerating assay system as given elsewhere [5]. For stopped flow experiments the enzyme protein was obtained from a stock solution and transferred to the appropriate buffer by spinning down the ammonium sulfate suspension of the enzyme, dissolving the pellet in the desired buffer and passing it through a Sephadex G-50 column preequilibrated with the same buffer. The enzyme was used immediately after this treatment in order to avoid significant activity loss occurring in the complete absence of ATP. Protein concentrations are based on a molecular weight of 400 000 [3]. ATP was obtained from Boehringer, Mannheim, ϵ-ATP from Sigma and AMP-PNP (β,γ-imidoadenosine-5′-triphosphate) as well as phenolred from Serva, Heidelberg. All other chemicals were of the purest grade commercially available. The concentrations of ATP, ϵ-ATP, AMP-PNP and phenolred were determined by absorbancy measurements in a Zeiss PMQ II photometer.

Stopped flow experiments using phenolred as indicator were carried out with an instrument described elsewhere [6]. The proton release was evaluated by converting the obtained absorbancy changes of the indicator at 560 nm to proton concentrations liberated in the reaction using the buffer-capacity of the system. The results were consistent with the end-point of the reaction as calculated from the total amount of ATP present in the reaction mixture. Experiments using the fluorescence change of ϵ-ATP Mn(II) were performed with a Durrum stopped flow instrument connected to a Digital Storage oscilloscope (Nicolet Instrument Corporation Model 1074) allowing signal averaging. Here, the quenching effect of Mn^{2+}-ions upon the fluorescence of ϵ-ATP allows one to follow the reaction [7]. ATP forms a more stable complex with Mn^{2+}-ions than ADP, thus changing the amount of nucleotide present as manganese complex at constant manganese-concentrations.

Since ϵ-ATP does not show any significant difference in the overall hydrolysis kinetics as compared to ATP it proved to be a most suitable substrate analogue in this technique [8]. The sample was excited at 300 nm and the emission was measured at > 390 nm. A linear relationship between fluorescence change and concentration was assumed, and the total fluorescence change did not exceed 5% in each experiment.

3. Results

The kinetics of the proton liberation process following a mixing of nearly equimolar concentrations of ATP and soluble ATPase in the presence of Mg-ions is shown in fig.1, revealing a lag-phase of 100 msec followed by a steady state hydrolysis of ATP. These results are confirmed in experiments at higher ϵ-ATP-concentrations in buffered solutions using a fluorescence intensity increase observed in the presence of ϵ-ATP as substrate and manganese as activating metal-ion. Figure 2 demonstrates an original record (a) obtained upon mixing of the enzyme and ϵ-ATP in the presence of manganese as well as the computed reaction course of the ϵ-ATP hydrolysis (b) and clearly indicates again the lag-phase of 100 msec. The application of both methods showed the relative independence of the lag-phase from the concentrations of ATP as well as protein.

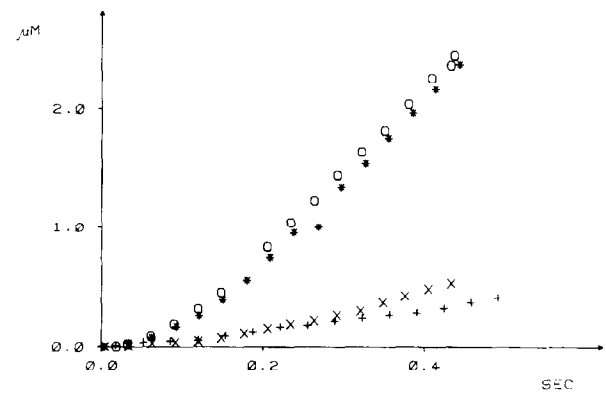

Fig.1. Proton release after mixing 4 μM ATPase with 20 (□, *) and 2 μM (X, +) ATP respectively in 5 mM magnesium acetate, 40 μM Tris-acetate, 25 μM phenolred and 30% glycerol. The change in the proton concentration was calculated from the measured absorbancy change at 560 nm (12°C, pH = 7,5, Δ pH = 0.1).

It is interesting to note that no rapid transient of a proton release could be observed, which might be attributed to the binding step of ATP to the enzyme. In order to support this observation AMP-PNP was used as the ATP-analogue which is bound but not hydrolyzed reacting as a strong competitive inhibitor of the ATPase reaction. Rapid mixing of the enzyme with this analogue did not yield a proton liberation step within the limit of the observation of 0.1 proton per active site. This finding is remarkable and in

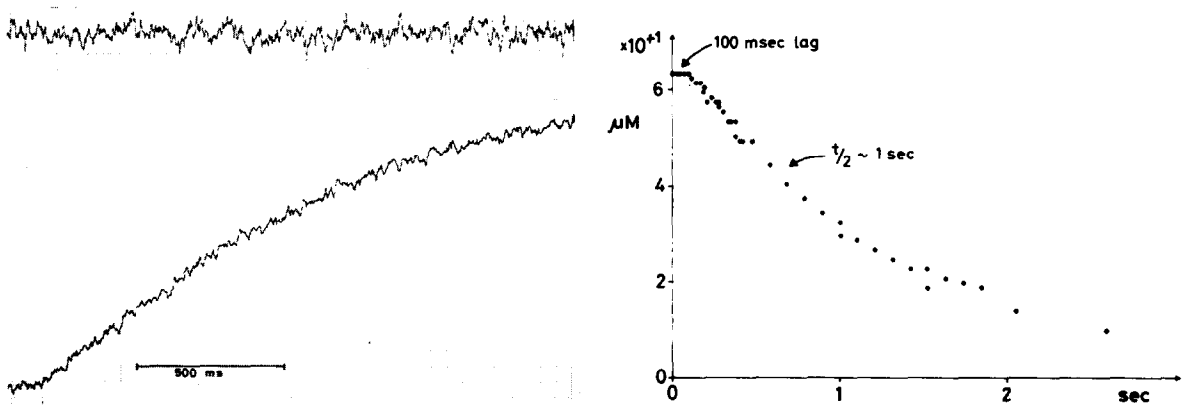

Fig.2. (a) Fluorescence change after mixing of 0.5 μM ATPase with 126 μM ϵ-ATP in 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-propane sulfonate K (HEPPS K), 5 mM $MnCl_2$ and 10% glycerol (pH = 7,8). Average of 4 experiments. Total fluorescence change about 5% (25°C). (b) Time course of ϵ-ATP-Mn(II)-hydrolysis by 0.25 μM ATPase (calculated from fig.2a).

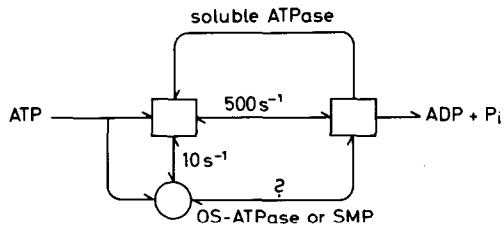

Fig.3. By-pass model for the ATPase reaction.

contrast to the observation reported for the ATP-binding step in myosin S_1 [9].

A computation of the turnover following the 100 msec lag-time of the ATPase-hydrolysis reaction yields turnover numbers in the range of 200 per sec corrected for only partial saturation and three catalytic sites, well within the range of the turnover number being observed during steady state experiments in the range of 300 sec^{-1}. This result clearly indicates that the slow conformation change observed as a lag-phase cannot be within the pathway of hydrolysis and leads to the assumption of a pre-equilibrium which turns the enzyme from a partially inactive state to an active state by a conformation change. ATP or the ATP metal complex might be the trigger for this relatively slow reaction.

An accompanying analysis of the transient kinetics of a crude ATPase preparation from Neurospora crassa (kindly provided by Dr Sebald) revealed a lag-phase of similar order as an initiation step of the hydrolysis reaction. Also, a lag-phase in the release of inorganic phosphate catalyzed by the ATPase of beef heart mitochondria was observed independently as recently reported by E. C. Slater using quenched flow techniques [10].

Whereas the slow conformation change does not fit the turnover number of soluble ATPase, it is in the order of the turnover number as observed in the case of OS-ATPase (oligomycin-sensitive ATPase) and the ATPase activity of submitochondrial particles, which in both cases is roughly 10 X lower [3]. We therefore suggest a bypass model as given in fig.3 describing the slow transition in the transient state experiments as a primary initiation event for the case of soluble ATPase where the catalysis would occur in a bypass which is controlled via rate controlling inhibitor units in the case of OS-ATPase, where the slow conformation change is part of the overall hydrolytic catalysis. Although it is suggested that this slow process is part of the overall ATP-synthase mechanism as given by Boyer and Slater [1,2], a definite conclusion cannot be drawn at the present time.

Acknowledgements

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