ADENYLYLIMIDODIPHOSPHATE RELEASE FROM THE ACTIVE SITE OF SUBMITOCHONDRIAL PARTICLES ATPase

B. C. CHERNYAK[+] and I. A. KOZLOV[++]

Department of Bioenergetics[+] and Isotope Department[++], A. N. Belozersky Laboratory of Molecular Biology and Bioorganic Chemistry, Moscow State University, Moscow 117234, U.S.S.R.

Received 29 May 1979

1. Introduction

Boyer and coworkers have shown that ATP release from the active site of H^+-ATPase is an energy-dependent stage of membrane-linked phosphorylation (see reviews [1,2]). According to Mitchell's chemiosmotic principle of energy coupling [3,4], transmembrane difference in the electrochemical potentials of hydrogen ions ($\Delta\bar{\mu}_{H^+}$) serves as the source of energy for ATP synthesis in the system of membrane-linked phosphorylation. Thus, it may be concluded that ATP release from the active site of H^+-ATPase occurs down the $\Delta\bar{\mu}_{H^+}$ gradient.

In our previous works we suggested that factor F_1 bearing the active site of H^+-ATPase is partially immersed in the mitochondrial membrane and thus lies in the electrical field that emerges in the membrane as a result of $\Delta\bar{\mu}_{H^+}$ generation [5–7]. The electrical forces facilitate the release of ATP from the active site of factor F_1 to the matrix [5,6].

To study the energy-dependent ATP release from the H^+-ATPase active site, in this work the non-hydrolysable analog of ATP, AMP-PNP was used. This compound, introduced into biochemical research by Yount et al. [8], is a potent inhibitor of both soluble and membrane-bound mitochondrial ATPase [9–17]. In this paper it is shown that the rate of AMP-PNP release from the active site of submitochondrial particles ATPase increases many times as a result of energisation of the membrane or a decrease in the pH of the incubation medium.

Abbreviations: Cl-CCP, *m*-chlorophenolcarbonylcyanide phenylhydrazone; AMP-PNP, adenylyl-5'-imidodiphosphate

2. Methods

Submitochondrial particles were obtained according to Hansen and Smith [18]; the ATPase activity of the particles was 0.2–0.4 μmol/min/mg protein at 25°C and 0.1–0.2 μmol/min/mg protein at 15°C. To determine the ATPase activity, H^+-ions release in the incubation medium during the ATPase reaction was measured [19]. The changes in pH during ATP hydrolysis were either registered by a glass pH-electrode or by measuring the changes in the optical density of the pH indicators. Neutral red (pH interval from 6.8 to 8.0, λ_{max} = 530 nm), cresol red (pH interval from 7.2 to 8.6, λ_{max} = 570 nm), and thymol blue (pH interval from 8.0 to 9.6, λ_{max} = 450 nm) were used as pH indicators. The changes in pH during the ATPase reaction did not exceed 0.1. The degree of substrate exhaustion during measurement of ATPase activity (10–12 min) did not exceed 5–10%. The dead time from the addition of the submitochondrial particles suspension to the reaction mixture to the start of measurement of the ATPase reaction rate was 15–20 sec in experiments with pH indicators, and 20–30 sec when the rate of ATP hydrolysis was measured by a pH electrode.

AMP-PNP and Cl-CCP were from Sigma. ATP was from Reanal (Hungary). Other chemical reagents were from Reachim (U.S.S.R.).

3. Results and discussion

Curve 1 in fig.1 shows the accumulation of the products of the ATPase reaction (in the given case

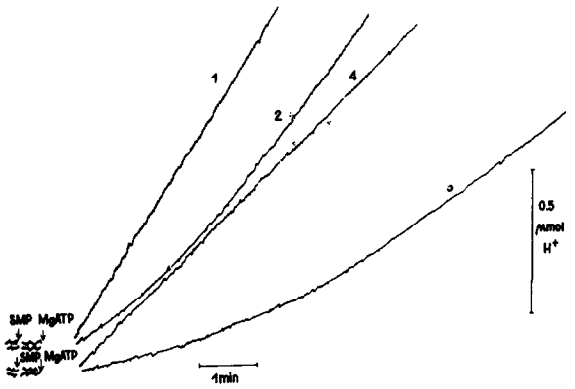

Fig.1. Reactivation of ATPase of submitochondrial particles preincubated with AMP-PNP. The particles (80 mg protein/ml) were preincubated in a mixture containing 0.25 M sucrose, 10 mM HEPES, pH 7.5, 100 μM $MgSO_4$ and 100 μM AMP-PNP (curves 2 and 3) for 5 min at 20°C. The control particles were preincubated in a mixture of the same composition, but in the absence of AMP-PNP and $MgSO_4$ (curves 1 and 4). 20 μl of the particles suspension in the preincubation mixture were added to the medium to measure the ATPase activity containing 0.25 M sucrose, 3 mM Tris–HCl, pH 8.3 and 2×10^{-6} M Cl-CCP in a volume of 8 ml. ATPase activity started with the addition to the solution of $MgSO_4$ + ATP (final concentration 2 mM). In the case of curves 1 and 2 measurement of ATPase activity was conducted at 27°C; curves 2 and 3 were obtained at 15°C.

H^+-ions) during the hydrolysis of ATP by submitochondrial particles. Beginning from the moment of registration of ATPase activity (30 sec after addition of Mg-ATP to the incubation medium) for 8 min the ATPase reaction rate remains constant. However, if the submitochondrial particles were preincubated with AMP-PNP, before addition to the medium to measure ATPase activity the initial ATP hydrolysis rate is considerably lower than the control one, and then for the next 2–3 min it grows and reaches a constant level, which is about 70% of the control (fig.1, curve 2). AMP-PNP is a strong inhibitor of mitochondrial ATPase ($K_i = 2 \times 10^{-7}$ M [13]). The AMP-PNP concentration in the medium to measure ATPase activity is many times less than in the medium where it was preincubated with the submitochondrial particles. The low activity of the particles at the initial moment of time and their subsequent low reactivation is evidently a result of the slow dissociation of the complex of AMP-PNP with ATPase. In the experiment shown in fig.1, curve 2, the time in which the particles reached half the maximum activity ($\tau_{1/2}$) was about 40 sec. The drop in temperature from 27 to 15°C leads to a slowing down of the reactivation process. In this case, $\tau_{1/2}$ is 2–2.5 min (fig.1, curve 3). Curve 4 in fig.1 shows that at 15°C, just as at 27°C, the particles not preincubated with AMP-PNP hydrolyse ATP at a constant rate.

The same picture as observed in fig.1 was obtained if ATP was present in the medium to measure ATPase activity at the beginning and the ATPase reaction was started by the addition of submitochondrial particles (not shown). In the experiment depicted in fig.1 pH electrodes were used to measure the ATPase activity. Similar results were obtained when pH indicators were used to measure the rate of the ATPase reaction. However, in the ATP regenerating system (in the presence of phosphoenolpyruvatekinase and phosphoenolpyruvate) the reactivation rate of the particles preincubated with AMP-PNP dropped sharply ($\tau_{1/2}$ >30 min; not shown). One gets the impression that ADP must be present in the incubation medium for AMP-PNP to be released from the active site of ATPase. This result is in good agreement

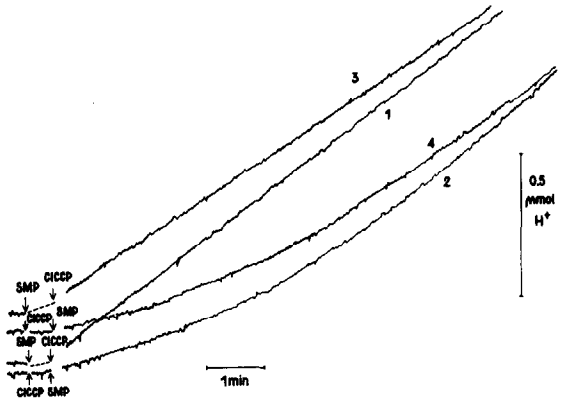

Fig.2. The effect of energisation on the reactivation rate of the particles preincubated with AMP-PNP. For the composition of the medium of preincubation of the particles with AMP-PNP, see fig.1. 20 μl of the suspension of particles preincubated with AMP-PNP were added to the medium to measure ATPase activity which contained 0.25 M sucrose, 3 mM Tris–HCl, pH 8.3, 2 mM ATP, 2 mM $MgSO_4$ and 5 mM succinate in an overall volume of 8 ml. In the case of curves 3 and 4 this medium also contained 50 mM KNO_3. 2×10^{-6} M Cl-CCP was added to the medium after the particles (curves 1 and 3) or before the particles (curves 2 and 4). Temperature 15°C.

with the data of Kayalar et al. [20], who showed that ATP release from the active site of mitochondrial ATPase is facilitated by the ADP binding with this enzyme.

Figure 2 shows the results of experiments in which the effect of membrane energisation on the reactivation rate of the particles preincubated with AMP-PNP was studied. Curve 1 was obtained when succinate and Mg-ATP were present in the incubation medium to which submitochondrial particles preincubated with AMP-PNP were added. 20 sec after the addition of the particles to this medium, an uncoupler, Cl-CCP, was added and measurement of ATPase activity started. Curve 2 was obtained in similar conditions; but besides succinate and Mg-ATP, the uncoupler was present in the incubation medium before the addition of the particles.

From the results obtained it can be seen that energisation of the particles preincubated with AMP-PNP leads to their rapid reactivation. The ATP hydrolysis rate measured 45 sec after the beginning of the ATPase reaction was maximal and remained constant for the next 10 min (fig.2, curve 1). The uncoupler, which abolishes succinate-induced energisation of particles membrane prevents their rapid reactivation (fig.2, curve 2). In this case, the maximum ATPase reaction rate was achieved in just 6 or 7 min. The ATPase activity of the control particles, which did not contain AMP-PNP, did not change as a result of 30-sec incubation with succinate in the experimental conditions shown in fig.2 (not shown).

The data obtained indicate that the energisation of particles membrane leads to a sharp increase in the rate of AMP-PNP release from the active site of ATPase. This result is in good agreement with the data of Melnick et al. [13], who showed that partial de-energisation of the particles membrane leads to a decrease in the constant of inhibition of ATPase by AMP-PNP. The results obtained justify our suggestion earlier [5,6] with regard to the reasons why AMP-PNP is a potent inhibitor of the ATPase activity of uncoupled particles and a weak inhibitor of the ATP synthetase reaction catalysed by coupled particles [10,12]. In actual fact, in conditions of membrane de-energisation the AMP-PNP release from the active site of mitochondrial ATPase to the solution occurs at a very low rate (fig.1, curves 2 and 3, and fig.2, curve 2); this ensures strong inhibition of ATPase activity even in the presence of high ATP concentrations. On the other hand, when the membrane is energised, dissociation of the ATPase complex with AMP-PNP occurs rapidly (fig.2, curve 1), and high concentrations of ADP and P_i may successfully compete with AMP-PNP for the active site of ATPase.

The increase in the rate of AMP-PNP release from the active site of ATPase as a result of membrane energisation is in good agreement with Boyer's concepts [1,2,20], according to which energisation of the membrane leads to a lowering of ATP affinity to the active site of ATPase.

The results obtained (fig.2) explain why de-energisation of the membrane by uncouplers leads to the inhibition of the ATP synthesis, even in conditions providing for a favourable shift of the reaction equilibrium (in the presence of hexokinase and glucose). In the absence of $\Delta\bar{\mu}_{H^+}$ on the membrane ATP release from the ATPase active site occurs very slowly and, despite the presence of the hexokinase trap, ATP synthesis becomes impossible.

The greater part of the energy of $\Delta\bar{\mu}_{H^+}$ generated on the mitochondrial membrane by respiration takes the form of transmembrane difference in the electrical potentials [21]. To elucidate the influence of the ΔpH-component of $\Delta\bar{\mu}_{H^+}$ on the rate of AMP-PNP release from the active site of ATPase, we studied the effect of the penetrating anion NO_3^- on the respiration-induced reactivation of the particles preincubated with AMP-PNP. It was previously shown [22] that 50 mM KNO_3, when added to the energised submitochondrial particles, leads to the complete disappearance of $\Delta\psi$ and to the emergence of ΔpH on the membrane.

Curve 3 in fig.2 shows that the succinate-induced energisation of particles membrane in the presence of 50 mM KNO_3 results in a sharp increase in the rate of reactivation of ATPase. By adding the uncoupler Cl-CCP to the medium with succinate and KNO_3 before the particles (curve 4), the effect of succinate was prevented. Thus, ΔpH on the membrane, as well as $\Delta\psi$, sharply increases the rate of AMP-PNP release from the active site of mitochondrial ATPase.

In the last series of experiments the pH-dependence of the rate of reactivation of particles preincubated with AMP-PNP was studied. It appeared that the lowering of pH leads to an increased rate of AMP-PNP release from the active site of ATPase. At

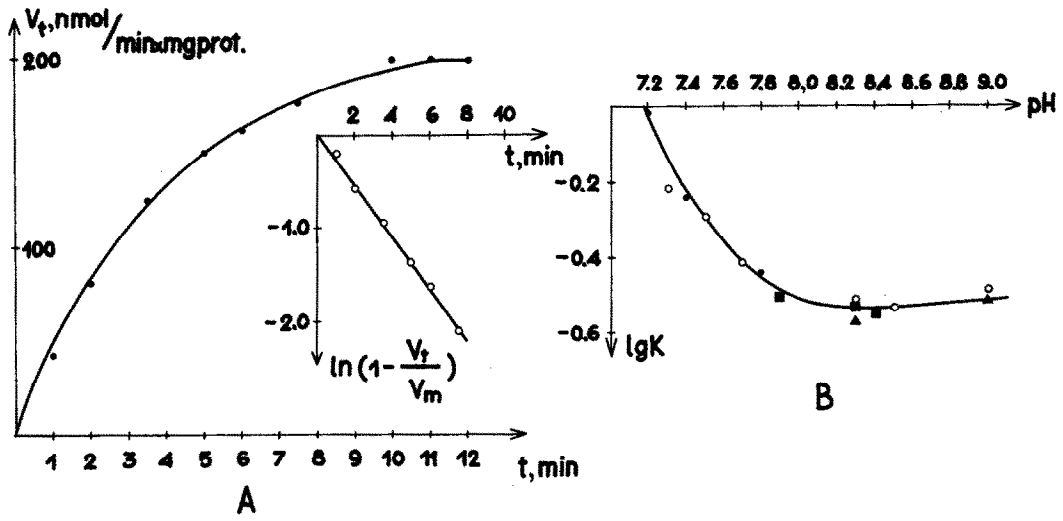

Fig.3. Determination of the dissociation rate constant of the complex of ATPase with AMP-PNP (A) and the pH-dependence of this constant (B). The particles were preincubated with AMP-PNP as in the legend to fig.1 and added to the medium to measure ATPase activity. A. The ATPase activity of submitochondrial particles preincubated with AMP-PNP, depending on the time of incubation in the medium to determine ATPase activity. Composition of the medium for ATPase activity measurements: 0.25 M sucrose, 2 mM Tris–HCl, pH 8.2, 2 mM ATP, 2 mM $MgSO_4$, 2×10^{-6} M Cl-CCP and 0.1 mg/ml cresol red in an overall volume of 3 ml. Temperature 13°C. The reaction was started by the addition of 5 μl (0.4 mg protein) of the particles. The ATPase activity was measured spectrophotometrically, using cresol red as the pH indicator. To determine the rate of the ATPase reaction at different moments of time, tangents to the curve showing the accumulation of the products of the ATPase reaction (H^+-ions) were drawn. Insert: the data obtained in the same experiment are given in semilogarithmic coordinates. V_t, the ATP hydrolysis rate at the moment t; V_{max}, maximum rate of ATP hydrolysis (200 nmol/min/mg protein at pH 8.2) after the reactivation process is completed. The tangent of the angle of inclination of the curve obtained is equal to the constant of the dissociation rate of the complex of ATPase with AMP-PNP (k). B. The ATPase activity was measured by a glass pH-electrode (o) or spectrophotometrically from the changes in the absorption of pH indicators neutral red (•); cresol red (▪); thymol blue (▲).

pH 7.0 reactivation occurs in less than 30 sec. $\tau_{1/2}$ reactivation at pH 7.2 was 40–50 sec. If reactivation of the particles is a result of AMP-PNP release from the active site of ATPase, this process can be described as a monomolecular reaction. In actual fact, at pH interval 7.2–9.0 the dependence of the ATPase activity of the particles on the incubation time is well described by the equation $V_t = V_m\,(1-e^{-kt})$ where V_t is the ATP hydrolysis rate at the moment in time t, V_m is the maximally possible hydrolysis rate, and k is the constant of the dissociation rate of the complex of ATPase with AMP-PNP. By way of example, fig.3A shows the dependence of ATPase activity of the particles precinubated with AMP-PNP on the time of incubation in the medium to measure the ATPase reaction rate at pH 8.2. Extrapolation of the time to zero gives the value $V_t = 0$, which points to the complete saturation of ATPase by the inhibitor, AMP-PNP, at the initial moment of time.

Figure 3B shows the pH dependence of the dissociation rate constant of the complex of ATPase with AMP-PNP. At any pH value the rate constants were measured in the same manner as is shown for the case of pH 8.2 in fig.3A. It can be seen that in the interval of the pH values from 7.2–7.9 the dissociation rate of the complex of ATPase with AMP-PNP drops to a minimum level and does not change any more as pH increases. It may be thought that the acceleration of AMP-PNP release from the active site of ATPase at low pH is the result of the protonation of a certain group of ATPase with $pK < 7.0$. It can be suggested that the protonation of this group by H^+ ions from the water phase inside the submitochondrial particles is the mechanism of the increase in the rate of ΔpH supported ATP synthesis on the mitochondrial membrane.

At the pH interval 8.0–9.0 the AMP-PNP release from the ATPase active site occurs at a low rate and

independent of pH (fig.3B). This means that the total rate of the AMP-PNP release is determined by the sum of the rates of the two processes, one of which depends on the protonation of the group with $pK < 7.0$, and the second does not depend on pH. When pH is increased to more than 8.0, this process independent of pH begins to make the main contribution to the dissociation rate of the complex of ATPase with AMP-PNP.

Thus, from the results obtained it follows that the ATP release from the active site of mitochondrial ATPase is accelerated manyfold by $\Delta\bar{\mu}_{H^+}$ generation on the membrane or by the decrease in the pH of the incubation medium.

Acknowledgements

The authors would like to thank Professor V. P. Skulachev for helpful discussion of the results and also Dr K. M. Shkrob for help in carrying out some of the experiments, and Mrs G. A. Kozlov for translation and preparation of the paper.

References

[1] Boyer, P. D. (1975) FEBS Lett. 58, 1–6.
[2] Boyer, P. D. (1977) Ann. Rev. Biochem. 46, 957–966.
[3] Mitchell, P. (1961) Nature 191, 144–148.
[4] Mitchell, P. (1966) Chemiosmotic Coupling in Oxidative and Photosynthetic Phosphorylation, Glynn Research, Bodmin.
[5] Kozlov, I. A. (1975) Bioorg. Khim. 1, 1545–1569.
[6] Kozlov, I. A., Skulachev, V. P. (1977) Biochim. Biophys. Acta 463, 29–89.
[7] Kozlov, I. A., Chernyak, B. V. (1976) Dokl. Acad. Nauk. SSSR, 231, 222–225.
[8] Yount, R. G., Babcock, D., Balantine, W., Ojala, D. (1971) Biochemistry 10, 2484–2489.
[9] Holland, P. C., LaBelle, W. L., Lardy, H. A. (1974) Biochemistry 13, 4549–4553.
[10] Penefsky, H. S. (1974) J. Biol. Chem. 249, 3579–3585.
[11] Philo, R. D., Selwyn, M. J. (1974) Biochem. J. 143, 745–749.
[12] Pederson, P. L., Le Vine, H. III, Cintron, N. (1974) in: Membrane Proteins in Transport and Phosphorylation, Amsterdam, North-Holland Publ. Co., pp. 43–54.
[13] Melnick, R. L., Tavares De Sousa, J., Maguire, J., Packer, L. (1975) Arch. Biochem. Biophys. 166, 139–144.
[14] Schuster, S. M., Ebel, R. E., Lardy, H. A. (1975) J. Biol. Chem. 250, 7848–7853.
[15] Garrett, N. E., Penefsky, H. S. (1975) J. Biol. Chem. 250, 6640–6647.
[16] Schuster, S. M., Gertschen, R. J., Lardy, H. A. (1976) J. Biol. Chem. 251, 6705–6710.
[17] Melnick, R. L., Donohue, T. (1976) Arch. Biochem. Biophys. 173, 231–236.
[18] Hansen, M., Smith, A. L. (1964) Biochim. Biophys. Acta 81, 214–222.
[19] Chance, B., Nishimura, M. (1967) Methods Enzymol. X, 641–690.
[20] Kayalar, C., Rosing, J., Boyer, P. D. (1977) J. Biol. Chem. 252, 2486–2491.
[21] Mitchell, P., Moyle, J. (1969) Eur. J. Biochem. 7, 471–484.
[22] Imedidze, E. A., Drobinskaya, I. E., Kerimov, T. M., Ruuge, E. K., Kozlov, I. A. (1978) FEBS Lett. 96, 115–119.