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The complex of mitochondrial F_1-ATPase with the natural inhibitor protein is unable to catalyze single-site ATP hydrolysis

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Interaction of mitochondrial F_1-ATPase with the isolated natural inhibitor protein resulting in the inhibition of multi-site ATP hydrolysis is accompanied by the loss of activity at low ATP concentrations when single-site hydrolysis should occur. Catalytic site occupancy by [^{14}C]nucleotides in F_1-ATPase during steady-state [^{14}C]ATP hydrolysis, which is saturated in parallel with single-site catalysis, is prevented after blocking the enzyme with the inhibitor protein.

F_1-ATPase; Inhibitor protein; Single-site catalysis

1. INTRODUCTION

The natural inhibitor protein (IP) of mitochondrial F_1-ATPase [1] is a physiological regulator of enzyme activity. IP suppresses the hydrolase activity of both isolated and membrane-bound F_1-ATPase but has no effect on the steady-state rate of ATP synthesis during oxidative phosphorylation (review [2,3]). Maximal inhibition of ATPase activity of both isolated F_1-ATPase [4,5] and the H^+-ATPase complex [5] is attained after binding of 1 mol of IP per mol of enzyme despite the fact that F_1-ATPase contains three active sites on the β-subunits.

F_1-ATPase can catalyze ATP hydrolysis by the single catalytic site (single-site catalysis) with the k_{cat} and K_m values of ~0.05 s^{-1} and ~10 nM, respectively [6–10] and with the sequential participation of two (or three, but only two of them are kinetically distinguished) catalytic sites (multi-site catalysis) [7,8,10]. Transition from single-site catalysis to a multi-site one results in ~10^4-fold acceleration of enzyme turnover [7,8,10]. This kinetic pattern of F_1-ATPase is in accord with the proposed alternating site mechanism [11,12]. There can be no doubt that multi-site catalysis is impaired by IP, though it is still unclear whether multi-site catalysis is completely prevented by IP or whether the IP·F_1-ATPase complex retains some ability to function in the multi-site mode. Meanwhile, it is unknown whether single-site catalysis can be affected by IP.

It is the aim of this study to resolve the latter question. The results presented indicate that not only multi-site catalysis but also single-site catalysis is destroyed in the IP·F_1-ATPase complex.

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Abbreviations: IP, natural inhibitor protein of mitochondrial F_1-ATPase; BSA, bovine serum albumin

2. MATERIALS AND METHODS

Tris and Mops from Sigma, BSA from Serva, Sephadex G-50 (fine) from Pharmacia, NADH, ATP, phospho*enol*pyruvate, pyruvate kinase and lactate dehydrogenase from Reanal, [U-^{14}C]ATP (440 mCi/mmol) from UVVVR and carrier free [γ-^{32}P]ATP (>1000 Ci/mmol) from Isotop were used.

F_1-ATPase was isolated from beef heart mitochondria according to Knowles and Penefsky [13]. IP was purified by the method of Frangione et al. [14] with minor modification. In the

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CM-Sephadex step IP was eluted with the solution containing 25 mM Tris-H_2SO_4 (pH 8.5) and 0.5 M Na_2SO_4.

To obtain the IP·F_1-ATPase complex, 0.25 μM F_1-ATPase was incubated for 20 min in the presence of 6 μM IP in a medium containing 0.25 M sucrose, 40 mM Mops-KOH (pH 6.8), 0.2 mM EDTA, 6 mM $MgCl_2$, 5 mM phospho*enol*pyruvate, 10 μM ATP and pyruvate kinase (1 mg/ml). Then 100 μl of the reaction mixture were applied to the precentrifuged column with 2 ml of Sephadex G-50 and eluted by centrifugation [15]. Sephadex was preequilibrated with the buffer containing 0.25 M sucrose, 50 mM Mops-KOH (pH 8.0), 2.5 mM $MgCl_2$, 0.2 mM EDTA, 5 mM P_i and 1.1 mg/ml of BSA. The procedure typically resulted in 80–90% inhibition of ATPase activity measured with 100 μM MgATP as substrate, but no inhibition was found in the absence of IP.

Multi-site ATPase activity was assayed by the spectrophotometric method [16] in the medium containing 20 mM Mops-KOH (pH 8.0), 60 mM KCl, 0.2 mM EDTA, 1 mM $MgCl_2$, 0.5 mM phospho*enol*pyruvate, 100 μM ATP, 2 mM Na_2SO_3, 0.3 mM NADH, pyruvate kinase (0.15 mg/ml) and lactate dehydrogenase (0.04 mg/ml). ATPase activity at low ATP concentrations (5–200 nM) was measured using [γ-^{32}P]ATP. The reaction was stopped with 3 M $HClO_4$ containing 6 mM P_i and unhydrolyzed [γ-^{32}P]ATP was removed by charcoal precipitation. For details see figure legend.

Catalytic site occupancy during [^{14}C]ATP hydrolysis in the presence of the ATP-regenerating system was investigated using the Sephadex column-centrifugation method [15] for separation of unbound nucleotide. The details are given in the legend to fig.2.

Protein was determined according to Lowry et al. [17] using BSA as a standard. A molecular mass of 360 kDa for F_1-ATPase and of 10.5 kDa for IP were used in calculations.

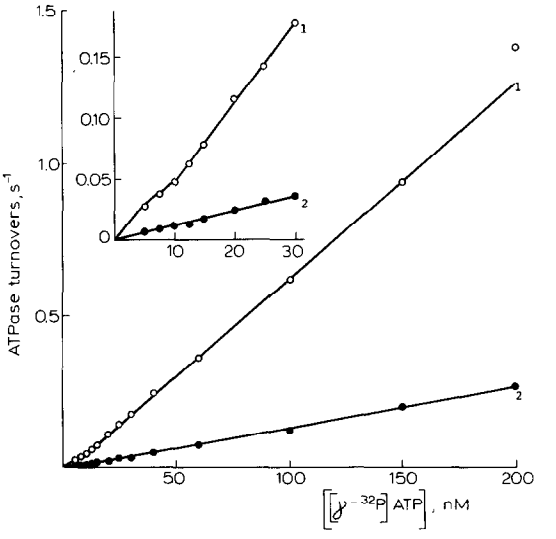

Fig.1. Hydrolysis of [γ-^{32}P]ATP by F_1-ATPase (curve 1, ○) or by IP·F_1-ATPase complex (curve 2, ●). The IP·F_1-ATPase complex was obtained as described in section 2. Hydrolysis of [γ-^{32}P]ATP (4.5 × 10^3 cpm/pmol at ATP concentrations ≤100 nM and 0.45 × 10^3 cpm/pmol at ATP concentrations >100 nM) was measured for 1 min in the medium containing 50 mM Mops-KOH (pH 8.0), 2.5 mM $MgCl_2$ and 0.2 mM EDTA. The concentration of F_1-ATPase was 0.5 and 1.0 nM and that of IP·F_1-ATPase complex was 2 and 4 nM at [γ-^{32}P]ATP concentrations < 10 nM and ≥10 nM, respectively. Inset shows the initial parts of curves 1 and 2.

3. RESULTS AND DISCUSSION

Curve 1 in fig.1 shows the dependence of the native F_1-ATPase activity on ATP concentration at substrate levels between 5 and 200 nM. This dependence is linear at ATP concentrations higher than 20 nM. The second-order rate constant for ATP binding derived from the linear portion of curve 1 (fig.1) is 6.4 × 10^6 $M^{-1}\cdot s^{-1}$ and coincides well to the values obtained under similar conditions by others [18,19]. The departure of curve 1 from a straight line at ATP concentrations lower than 20 nM is due to the alteration of the pattern of ATP hydrolysis by F_1-ATPase from a multi-site regime to single-site catalysis.

Preincubation of F_1-ATPase in the presence of 10 μM ATP and the ATP-regenerating system at pH 6.8 for 20 min does not affect the enzyme activity assayed with 100 μM ATP as a substrate and has no influence on the ATP dependence of F_1-ATPase activity at substrate concentrations ranging from 5 to 200 nM (not shown). However, the addition of 6 μM IP to the preincubation medium leads to the decrease of ATPase activity at 100 μM ATP by about 85% and to corresponding inactivation at low substrate concentrations (fig.1, curve 2). The residual activity is likely to result from F_1-ATPase molecules not containing IP. These results indicate that, preventing the multi-site activity of F_1-ATPase, IP also inhibits the single-site catalysis. To verify this conclusion we investigated the catalytic site occupancy in the IP·F_1-ATPase complex in the presence of ATP and the ATP-regenerating system.

It was recently shown by our group that during steady-state ATP hydrolysis by the nucleotide-depleted F_1-ATPase, the enzyme contains about 1 mol of transitorily bound nucleotides (ADP + ATP) in the catalytic sites [20]. Similar results were obtained for membrane-bound F_1-ATPase [10]. The extent of the catalytic site occupancy by the transitorily bound nucleotides in F_1-ATPase coin-

cides well with the extent of saturation of single-site catalysis by the substrate [7,8,10].

It may be seen in fig.2 (curve 1) that native F_1-ATPase during steady-state [^{14}C]ATP hydrolysis binds up to 1.2 mol of [^{14}C]adenine nucleotides per mol of enzyme. 80–90% of the bound [^{14}C]nucleotides are removed if the separation of free nucleotide is carried out on the columns equilibrated with the medium containing 1 mM cold ATP. Raising the [^{14}C]ATP concentration up to 2 μM has no influence on the stoichiometry of the transitorily bound [^{14}C]nucleotides. Half-maximal occupancy of catalytic site by [^{14}C]nucleotides occurs at a [^{14}C]ATP concentration of about 5 nM (fig.2, curve 1).

The pattern of the catalytic site occupancy during [^{14}C]ATP hydrolysis does not change after the preincubation of F_1-ATPase at pH 6.8 in the presence of ATP and the ATP-regenerating system (not shown). Meanwhile, as shown by curve 2 (fig.2), the inactive IP·F_1-ATPase complex is incapable of binding [^{14}C]nucleotides at the catalytic site in the presence of [^{14}C]ATP. This result confirms the conclusion that the inactive IP·F_1-ATPase complex in multi-site catalysis is also incapable of single-site catalysis.

Recently Dunn et al. [21] have shown that the ϵ-subunit of *Escherichia coli* F_1-ATPase, which is similar to the mitochondrial IP in inhibiting the F_1-ATPase but is homologous to the δ-subunit of bovine enzyme [22], inhibits single-site and tri-site ATP hydrolysis by *E. coli* F_1-ATPase but not the bi-site one. However, Wood et al. [23] have found that the extent of intermediate P_i-water oxygen exchange during ATP hydrolysis is increased by the ϵ-subunit. If the ATP dependence of the intermediate exchange reflects the acceleration of the product release step at the first catalytic site upon the binding of ATP at the second alternating catalytic site, the conclusion of Dunn et al. [21] that the ϵ-subunit does not inhibit the bi-site ATP hydrolysis is in conflict with the results of Wood et al. [23]. It is possible that this discrepancy is due to the fact that the process with very slow product release, which in the case of mitochondrial F_1-ATPase was shown not to be carried out by the normal catalytic site [9,19], is considered by Dunn et al. [21] as the single-site hydrolysis.

To explain the results obtained in the present

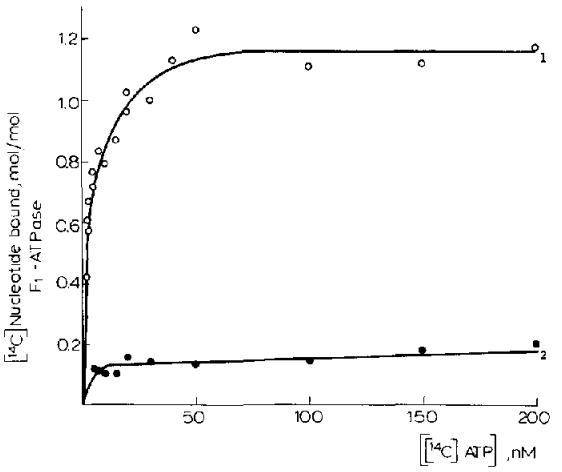

Fig.2. Catalytic site occupancy in the native F_1-ATPase (curve 1, ○) or in the IP·F_1-ATPase complex (curve 2, ●). F_1-ATPase or the IP·F_1-ATPase complex, obtained as described in section 2, were incubated in the presence of [^{14}C]ATP in a medium containing 50 mM Mops-KOH (pH 8.0), 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM phospho*enol*pyruvate, 1 mg/ml pyruvate kinase and 1.1 mg/ml BSA for sufficient time to allow several turnovers (from 5 min at 2 nM ATP to 0.5 min at $\geq$100 nM ATP). Unbound nucleotide was removed by column centrifugation [15] using Sephadex G-50 equilibrated with the same medium but lacking pyruvate kinase. The concentration of F_1-ATPase and IP·F_1-ATPase complex was 5–50-fold lower than the [^{14}C]ATP concentration.

work, two mechanisms may be proposed. (i) The steps of ATP binding and/or splitting in the catalytic site are impaired in the IP·F_1-ATPase complex. (ii) In the inactive IP·F_1-ATPase complex, the product (ADP or P_i, or both) release step and, probably, the catalysis promoting step after ATP binding at the second catalytic site are greatly inhibited (or totally prevented). A mechanism similar to (ii) has been proposed for F_1-ATPase modified by dicyclohexylcarbodiimide and 7-chloro-4-nitrobenzofurazan [24], though in the latter case, ATP binding was found to be affected also [19]. Mechanism (ii) seems to be more probable for the inhibition of F_1-ATPase by IP, since an incubation in the presence of hydrolyzable substrate is required to obtain the inactive IP·F_1-ATPase complex (review [3]). It may be speculated that nucleotide is trapped in one of the catalytic sites of the inactive IP·F_1-ATPase complex. This suggestion is currently under investigation.

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REFERENCES

[1] Pullman, M.E. and Monroy, G.C. (1963) J. Biol. Chem. 238, 3762–3769.
[2] Pedersen, P.L., Schwerzmann, K. and Cintron, N. (1981) Curr. Top. Bioenerg. 11, 149–199.
[3] Schwerzmann, K. and Pedersen, P.L. (1986) Arch. Biochem. Biophys. 250, 1–18.
[4] Klein, G., Satre, M., Dianoux, A.-C. and Vignais, P.V. (1980) Biochemistry 19, 2919–2925.
[5] Wong, S.-Y., Galante, Y.M. and Hatefi, Y. (1982) Biochemistry 21, 5781–5787.
[6] Kozlov, I.A., Milgrom, Ya.M. and Murataliev, M.B. (1986) Biol. Membranes (USSR) 3, 43–51.
[7] Milgrom, Ya.M. and Murataliev, M.B. (1986) Biol. Membranes (USSR) 3, 890–905.
[8] Milgrom, Ya.M. and Murataliev, M.B. (1987) FEBS Lett. 212, 63–67.
[9] Milgrom, Ya.M. and Murataliev, M.B. (1987) FEBS Lett. 222, 32–36.
[10] Milgrom, Ya.M. and Murataliev, M.B. (1987) Biol. Membranes (USSR) 4, 1180–1188.
[11] Repke, K.R.H. (1977) in: Biochemistry of Membrane Transport, FEBS Symposium 42 (Semenza, G. and Carafoli, E. eds) pp.363–373, Springer, Berlin.
[12] Boyer, P.D. (1979) in: Membrane Bioenergetics (Lee, C.-P. et al. eds) pp.461–479, Addison-Wesley, Reading, MA.
[13] Knowles, A.F. and Penefsky, H.S. (1972) J. Biol. Chem. 247, 6617–6623.
[14] Frangione, B., Rosenwasser, E., Penefsky, H.S. and Pullman, M.E. (1981) Proc. Natl. Acad. Sci. USA 78, 7403–7407.
[15] Penefsky, H.S. (1977) J. Biol. Chem. 252, 2891–2899.
[16] Pullman, M.E., Penefsky, H.S., Datta, A. and Racker, E. (1960) J. Biol. Chem. 235, 3322–3329.
[17] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[18] Cross, R.L., Grubmeyer, C. and Penefsky, H.S. (1982) J. Biol. Chem. 257, 12101–12105.
[19] Bullough, D.A., Verburg, J.G., Yoshida, M. and Allison, W.S. (1987) J. Biol. Chem. 262, 11675–11683.
[20] Milgrom, Ya.M. and Murataliev, M.B. (1986) Biol. Membranes (USSR) 3, 981–983.
[21] Dunn, S.D., Zadorozny, V.D., Tozer, R.G. and Orr, L.E. (1987) Biochemistry 26, 4488–4493.
[22] Walker, J.E., Fearnley, I.M., Gay, N.J., Gibson, B.W., Northrop, F.D., Powell, S.J., Runswick, M.J., Saraste, M. and Tybulewicz, V.L.J. (1985) J. Mol. Biol. 184, 677–701.
[23] Wood, J.M., Wise, J.G., Senior, A.E., Futai, M. and Boyer, P.D. (1987) J. Biol. Chem. 262, 2180–2186.
[24] Kandpal, R.P., Melese, T., Stroop, S.D. and Boyer, P.D. (1985) J. Biol. Chem. 260, 5542–5547.