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Mechanism of ATP Synthesis by Mitochondrial ATP Synthase from Beef Heart

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Previous studies of the rate constants for the elementary steps of ATP hydrolysis by the soluble and membrane-bound forms of beef heart mitochondrial F_1 supported the proposal that ATP is formed in high-affinity catalytic sites of the enzyme with little or no change in free energy and that the major requirement for energy in oxidative phosphorylation is for the release of product ATP.

The affinity of the membrane-bound enzyme for ATP during NADH oxidation was calculated from the ratio of the rate constants for the forward binding step (k_{+1}) and the reverse dissociation step (k_{-1}). k_{-1} was accelerated several orders of magnitude by NADH oxidation. In the presence of NADH and ADP an additional enhancement of k_{-1} was observed. These energy-dependent dissociations of ATP were sensitive to the uncoupler FCCP. k_{+1} was affected little by NADH oxidation. The dissociation constant ($K_{d\,ATP}$) increased many orders of magnitude during the transition from nonenergized to energized states.

KEY WORDS: ATPase; oxidative phosphorylation; mechanism of energy conversion in mitochondria.

The transfer of reducing equivalents in the electron transport chain of mitochondria can generate vectorial protons capable of supporting ATP synthesis (Mitchell, 1961). As originally proposed by P. D. Boyer, energy made available by proton translocations can be utilized to alter conformational states of the F_0F_1 ATPase that result in ATP formation and release (for a review see Boyer, 1993). This review discusses changes in the catalytic sites of the F_0F_1 ATPase that can account for the reactions of ATP synthesis.

W. P. Jencks has emphasized the role of the intrinsic binding energy as a driving force in catalysis (Jencks, 1975, 1989). According to Jencks, high-affinity binding of ATP in the active site of an enzyme "pulls" the equilibrium toward ATP formation (Jencks, 1989). In contrast, binding of ADP and Pi is relatively weak. The weak binding of ADP and Pi can be related to destabilization forces in the catalytic site. Release of these forces favors ATP formation (Jencks, 1989). Thus, the energy difference between tight binding of ATP and loose binding of ADP and Pi (the interaction energy) is a critical factor in the formation of ATP (Jencks, 1989). The enzyme–substrate complex (see below) represents a higher energized state, so that ADP and Pi can readily react to form ATP (Jencks, 1989).

Based on the observation that the Pi $\rightleftharpoons$ HOH oxygen exchange reaction catalyzed by submitochondrial particles was less sensitive to uncouplers, Boyer and coworkers concluded that the reversible formation of ATP was independent of proton flux (Boyer *et al.*, 1973). In contrast, the Pi $\rightleftharpoons$ ATP exchange, a reaction requiring dissociation and rebinding of ATP, was highly sensitive to uncouplers. This observation raised the possibility that the energy of proton transductions is required, at least in part, for release of bound ATP (Boyer *et al.*, 1973; Boyer, 1993).

Unisite catalysis is defined as ATP hydrolysis in a

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single catalytic site of multisite ATPases (for review see Penefsky and Cross, 1991; Penefsky, 1986). Catalysis at a single site of the enzyme is characterized by the following properties: (a) The affinity of the site for ATP ($K_{a\,ATP}$) is very high, $10^{12}\,M^{-1}$. (b) Negative cooperativity in binding. (c) The rate of hydrolysis of ATP in a single catalytic site is slow; the turnover number is $10^{-3}\,s^{-1}$. (d) Cooperativity between catalytic sites; ATP hydrolysis is accelerated to $600\,s^{-1}$ (as much as five orders of magnitude) when substrate is made available to additional sites on the enzyme (Penefsky and Cross, 1991). The elementary steps for ATP hydrolysis in a single catalytic site of soluble beef heart F_1 (Cross *et al.*, 1982; Grubmeyer *et al.*, 1982) and membrane-bound (F_0F_1) (Penefsky, 1985a) were determined under unisite conditions. These steps are summarized below:

$$F_1 + ATP \overset{\text{Step 1}}{\rightleftharpoons} F_1 \cdot ATP \overset{\text{Step 2}}{\rightleftharpoons} F_1 \cdot ADP \cdot Pi$$

$$\overset{\text{Step 3}}{\underset{\text{Pi}}{\rightleftharpoons}} F_1 \cdot ADP \overset{\text{Step 4}}{\underset{\text{ADP}}{\rightleftharpoons}} F_1$$

Step 1 represents the formation of the enzyme–substrate complex ($F_1 \cdot ATP$). The bimolecular rate constant describing the binding of [γ-^{32}P]ATP by F_1, the "on" rate (k_1), is fast ($6 \times 10^6\,M^{-1}\,s^{-1}$). However, the first-order rate constant describing [γ-^{32}P]ATP dissociation from the enzyme–substrate complex, the "off" rate (k_{-1}), is slow ($7 \times 10^{-6}\,s^{-1}$). The dissociation constant (that is, the equilibrium constant, $K_{d\,ATP}$) for ATP bound in a high-affinity catalytic site, calculated from the ratio of the reverse (k_{-1}) and forward (k_{+1}) rate constants is $10^{-12}\,M$. Thus, under unisite conditions, addition of [γ-^{32}P]ATP to F_1 is followed by rapid binding of added ATP and virtually no dissociation.

Step 2 is the catalytic step. Products remain bound in the catalytic site. The equilibrium constant (K_2) is near unity, suggesting that this step takes place with little or no change in Gibbs free energy.

Steps 3 and 4 are product release steps. Pi and ADP dissociate from the enzyme at slow and apparently equal rates. k_{-3} and k_{-4} are $4 \times 10^{-3}\,s^{-1}$ (Cunningham and Cross, 1988). These latter rates define the net rate of hydrolysis under unisite conditions.

The properties of unisite catalysis, as described above, support the binding change mechanism (Boyer, 1993; Rosing *et al.*, 1977; Kayalar *et al.*, 1977). Reversible ATP formation takes place with little or no change in Gibbs free energy, is not coupled to proton translocation, and is insensitive to uncouplers. The release of product ATP is the energy-requiring step in the ATP synthesis. Binding of ADP and Pi are energy-requiring reactions (Boyer, 1993).

The energy-dependent release of ATP bound in high-affinity catalytic sites of submitochondrial particles from beef heart was studied under unisite conditions during oxidation of reduced respiratory chain substrates (Penefsky, 1985b). Rapid rates of ATP dissociation were observed. The rates were commensurate with the rate of ATP synthesis catalyzed by the same particles (Penefsky, 1985b). The NADH-stimulated dissociation of [γ-^{32}P]ATP was prevented by either FCCP or oligomycin (Penefsky, 1985b).

These properties of the high-affinity catalytic site of beef heart mitochondrial ATPase support the conclusion that this site is a competent catalytic site. It participates in hydrolysis at V_{max} rates, is accessible to cooperative interactions between catalytic subunits, and can utilize energy released during proton flux (Penefsky, 1985b; Penefsky and Cross, 1991). Unisite catalysis closely similar to that of beef heart mitochondria has been described for soluble F_1 from years mitochondria (Mueller, 1989) and *Escherichia coli* (Noumi *et al.*, 1986; Al Shawi and Senior, 1988). There were also similarities in chloroplast F_0F_1-type ATPase (Graber, 1986). Yohda and Yoshida (1987) found little or no evidence for unisite catalysis in the ATPase of the thermophilic bacterium PS3 when ATP was used as a substrate. However, an acceleration in the hydrolysis of trinitrophenyl-ATP (TNP-ATP) was observed when the enzyme was switched from conditions of unisite to multisite catalysis (Hisabori *et al.*, 1992). Such an acceleration is diagnostic of unisite catalysis (Penefsky and Cross, 1991).

An analysis of the soluble F_1 ATPase from *E. coli* plasma membranes found that enzyme preparations lacking the δ subunit exhibited the rate accelerations characteristic of unisite hydrolysis of ATP while enzyme preparations containing a full complement of the δ subunit did not show such accelerations (Xiao and Penefsky, 1994). Reconstitution of a 5-subunit enzyme by incubating 4-subunit preparations (lacking the δ subunit) with a purified preparation of subunit δ was accompanied by disappearance of the response to a cold chase. Because the δ subunit was required for binding of the soluble enzyme to F_1-depleted vesicles (Bragg *et al.*, 1973; Futai *et al.*, 1974; Sternweis and Smith, 1977; Engelbrecht and Junge, 1990; Mendel-Hartvig and Capaldi, 1991), it

might be expected that membrane-bound *E. coli* F_0F_1 would not exhibit rate accelerations characteristic of unisite catalysis. An absence of cold-chase acceleration of ATP hydrolysis by *E. coli* vesicles was in fact noted. However, after extraction of the vesicles with KCl, cold-chase accelerations were observed and moreover, ATP bound in high-affinity catalytic sites of membrane-bound *E. coli* F_1 was subject to energy-dependent dissociation (Xiao and Penefsky, 1994).

Recently, we examined the kinetic properties of F_0F_1-ATPase from beef heart mitochondria during NADH oxidation in the presence and absence of ADP (Souid and Penefsky, 1994). The first-order rate constant for dissociation of bound [γ-^{32}P]ATP from high-affinity catalytic sites of submitochondrial particles (k_{-1}) was accelerated several orders of magnitude by NADH oxidation. In the presence of NADH and ADP, an additional enhancement in k_{-1} was observed. The bimolecular rate constant for binding of ATP to high-affinity catalytic sites of the enzyme (k_{+1}) was affected little if at all by NADH or NADH and ADP. The dissociation constant ($K_{d\,ATP}$) increased more than seven orders of magnitude during the transition from nonenergized to energized states (Souid and Penefsky, 1994).

The ADP-promoted dissociation of ATP was observed only during respiration and was sensitive to FCCP (Souid and Penefsky, 1994). Thus, part of the energy of respiration was utilized by the enzyme to bring about cooperative interactions between subunits, facilitating product release (Souid and Penefsky, 1994). Cooperative interactions also were observed during ATP hydrolysis (Grubmeyer and Penefsky, 1981a,b) and ATP synthesis (Matsuno-Yagi and Hatefi, 1985). Addition of excess ATP (cold chase) or ADP promoted the hydrolysis of bound [γ-^{32}P]ATP in nonenergized membranes and the dissociation of bound [γ-^{32}P]ATP in energized membranes (Souid and Penefsky, 1994). Thus, cooperative forces are directed toward facilitating product ATP release during respiration (Souid and Penefksy, 1994).

To illustrate the discussion, a representation of a reaction mechanism for ATP synthesis in accord with the concepts of binding energy, binding change mechanism, and unisite catalysis is shown in Fig. 1. The catalytic steps at a single site are shown in A. The energy level of the conformational states of a catalytic site during ATP synthesis is shown in B. Possible conformational states of the F_0 complex are presented in C. The first step of the forward reaction

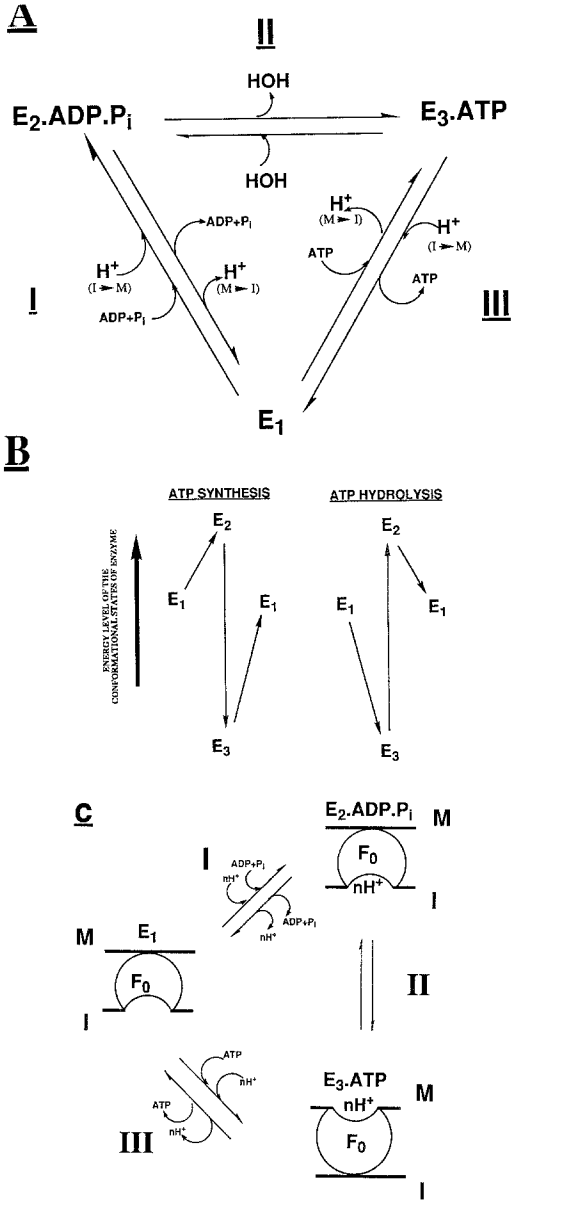

Fig. 1. Reaction Mmechanism for ATP Synthesis. (A) The catalytic steps at a single site. (B) The energy level of the conformational states of a catalytic site during ATP synthesis and hydrolysis. (C) Possible conformational states of the F_0 complex.

(ATP synthesis) couples binding of ADP and Pi to proton translocation from the intermembrane space (I) to the mitochondrial matrix (M) (Fig. 1A). The captured energy of proton translation is utilized to overcome the loss of entropy of binding of ADP and Pi (Jencks, 1989). This energy is stored in a higher-energized state of the enzyme, E_2 (Fig. 1B, ATP synthesis). Destabilization forces are responsible for the energized $E_2 \cdot ADP \cdot Pi$ complex

(Fig. 1B). Release of these forces favors ATP formation (Jencks, 1989). In step II, the reversible formation of ATP occurs independent of proton translocation, that is, the reaction is insensitive to uncouplers. During synthesis, ATP formation is driven by the free energy of binding of product ATP. Tight binding of ATP occurs in the high-affinity catalytic site observed during unisite catalysis (Fig. 1B, ATP hydrolysis). In step III, the energy of proton translocation from I to M is captured to lower the affinity of the enzyme for ATP, thus promoting its release. Proton translocation from I to M requires that ATP formation should take place at the catalytic sites. In the absence of net phosphorylation (due to, for example, ADP depletion) generated protons fail to translocate and inhibition of respiration supervenes (respiratory control). The formation energy (equivalent to binding energy) of ATP may be captured by F_0 in conformational changes that are necessary for proton translocation (Fig. 1C). Each of these catalytic steps, including those which are independent of proton translocation, can be inhibited by compounds such as oligomycin or dicyclohexylcarbodiimide, both of which bind to the F_0 portion of the F_0F_1 complex. This observation supports the suggestion of long-range interactions between subunits of the enzyme during ATP synthesis (Penefsky, 1985c).

The first step in ATP hydrolysis is the binding of ATP in high-affinity catalytic sites of the enzyme (Fig. 1B, ATP hydrolysis). The free energy of binding of ATP is coupled to proton translocation from M to I. In step II, the reversible hydrolysis of ATP is independent of proton translocation (Fig. 1A). Release of ADP and Pi (step III) relieves the destabilization forces and regains the loss of entropy of bound ADP and Pi. These reactions drive the net movement of protons from M to I (Fig. 1B).

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REFERENCES

Al Shawi, M. K., and Senior, A. E. (1988). *J. Biol. Chem.* **263,** 19640–19648.
Bragg, P. D., Davies, P. L., and Hou, C. (1973). *Arch. Biochem. Biophys.* **159,** 664–671.
Boyer, P. D. (1993). *Biochim. Biophys. Acta* **1140,** 215–250.
Boyer, P. D., Cross, R. L., and Momsen, W. (1973). *Proc. Natl. Acad. Sci. USA* **70,** 2837–2839.
Cross, R. L., Grubmeyer, C., and Penefsky, H. S. (1982). *J. Biol. Chem.* **257,** 12101–12105.
Cunningham, D., and Cross, R. L. (1988). *J. Biol. Chem.* **263,** 18850–18856.
Engelbrecht, S., and Junge, W. (1990). *Biochim. Biophys. Acta* **1015,** 379–390.
Futai, M., Sternweiss, P. C., and Heppel, L. A. (1974). *Proc. Natl. Acad. Sci. USA* **71,** 2725–2729.
Graber, P., Fromme, P., Junesch, U., Schmidt, G., and Thulk, G. (1986). *Ber. Bunsenges. Phys. Chem.* **90,** 1034–1040.
Grubmeyer, C., and Penefksy, H. S. (1981a). *J. Biol. Chem.* **256,** 3718–3727.
Grubmeyer, C., and Penefsky, H. S. (1981b). *J. Biol. Chem.* **256,** 3728–3734.
Grubmeyer, C., Cross, R. L., and Penefsky, H. S. (1982). *J. Biol. Chem.* **257,** 12092–12100.
Hisabori, T., Muneguki, E., Odaka, M., Yokoyama, K., Mochizuki, K., and Yoshida, M. (1992). *J. Biol. Chem.* **267,** 4551–4556.
Jencks, W. P. (1975). *Adv. Enzymol.* **43,** 219–410.
Jencks, W. P. (1989). *Methods Enzymol.* **171,** 145–164.
Kayalar, C., Rosing, J., and Boyer, P. D. (1977). *J. Biol. Chem.* **252,** 2486–2491.
Matsuno-Yagi, A., and Hatefi, Y. (1985). *J. Biol. Chem.* **260,** 14424–14427.
Mendel-Hartvig, J., and Capaldi, R. A. (1991). *Biochim. Biophys. Acta* **1060,** 115–124.
Mitchell, P. (1961). *Nature (London)* **191,** 144–148.
Mueller, D. M. (1989). *J. Biol. Chem.* **264,** 16552–16556.
Noumi, T., Taniai, M., Kanazawa, H., and Futai, M. (1986). *J. Biol. Chem.* **261,** 9196–9201.
Penefsky, H. S. (1985a). *J. Biol. Chem.* **260,** 13728–13734.
Penefsky, H. S. (1985b). *J. Biol. Chem.* **260,** 13735–13741.
Penefsky, H. S. (1985c). *Proc. Natl. Acad. Sci. USA* **82,** 1589–1593.
Penefsky, H. S. (1986). *Methods Enzymol.* **126,** 608–618.
Penefsky, H. S., and Cross, R. L. (1991). *Adv. Enzymol. Relat. Areas Mol. Biol.* **64,** 173–214.
Rosing, J., Kayalar, C., and Boyer, P. D. (1977). *J. Biol. Chem.* **252,** 2478–2485.
Souid, A. K., and Penefsky, H. S. (1994). Unpublished observations.
Sternweis, P. C., and Smith, J. B. (1977). *Biochemistry* **16,** 4020–4025.
Xiao, R., and Penefsky, H. S. (1994). *J. Biol. Chem.*, in press.
Yohda, M., and Yoshida, M. (1987). *J. Biochem.* **269,** 4233–4239.