Communication

Studies on the Mechanism of Oxidative Phosphorylation

CATALYTIC SITE COOPERATIVITY IN ATP SYNTHESIS*

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Oxidative phosphorylation catalyzed by bovine heart submitochondrial particles appears to exhibit negative cooperativity with respect to [ADP] and positive cooperativity in catalysis. Eadie-Hofstee plots \( (v/[S] \text{ versus } v) \) of the kinetics of oxidative phosphorylation at the variable ADP concentration range of 1-1200 \( \mu M \) were curvilinear and could be analyzed for two apparent \( K_{\text{app}} \) values differing by one order of magnitude, and two apparent \( V_{\text{max}} \) values. The \( K_{\text{app}} \) values with either NADH or succinate as the respiratory substrate were in the ranges of 10 and 100 \( \mu M \), and the \( V_{\text{max}} \) values in nmol of ATP formed-min\(^{-1} \) (mg of protein\(^{-1} \)) were, respectively, 500 and 1840 when NADH was the oxidizable substrate, and 550 and 100 when succinate was the energy source. Site-site cooperativity of the ATP synthase, which is a central feature of current theories for the mechanism of oxidative phosphorylation, has been well-documented for ATP hydrolysis by isolated F\(_{1}\)-ATPase, but never before demonstrated for mitochondrial ATP synthase.

*F\(_{1}\)-ATPase from bovine heart mitochondria is composed of five unlike subunits with the stoichiometric ratio of \( \alpha_3 \beta_2 \gamma_3 \delta_4 \) (1, 2). The \( \beta \) subunits alone or in combination with the \( \alpha \) subunits carry the catalytic sites of the enzyme (1, 2). In a system containing an F\(_{1}\)-ATP molar ratio of 3, Penefsky and co-workers (3, 4) were able to demonstrate that a single catalytic site of the isolated F\(_{1}\)-ATPase is capable of ATP hydrolysis, albeit at an extremely slow rate \( (10^{-2} \text{ s}^{-1}) \). At physiological ATP concentrations, F\(_{1}\) exhibits negative cooperativity with respect to [ATP] (3-7), and positive catalytic cooperativity in the sense that ATP binding to a second and a third site greatly enhances enzyme turnover apparently by increasing the rate of product (ADP) removal from the first site (4, 7). Consistent with the view that each F\(_{1}\)-ATPase molecule contains three functional and interacting catalytic sites, it has been shown that the curvilinear Eadie-Hofstee plots \( (v/[S] \text{ versus } v) \) of ATP hydrolysis by isolated bovine heart F\(_{1}\)-ATPase represent three apparent \( K_{\text{app}} \) and three associated \( V_{\text{max}} \) values, the former being of the order of \( 10^{-2} \)-

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\( 10^{-4}, 10^{-4}, \) and \( 10^{-3} \) M, and the latter (per min per mg of protein) being in the range of micromoles or less for the lowest \( K_{\text{app}} \) and decamicromoles for the other two (8).

Site-site cooperativity in F\(_{1}\)-ATPase is a central feature of Boyer's proposed binding change mechanism for oxidative phosphorylation (7, 9-11). However, clear evidence of site-site cooperativity in the direction of ATP synthesis has been lacking. Recently, Stroop and Boyer (12) have published data suggesting cooperativity in photophosphorylation and have estimated apparent \( K_{\text{app}} \) values of 0.62 and 31 \( \mu M \) and respective \( V_{\text{max}} \) values of 37 and 907 \( \mu \text{mol} \cdot \text{h}^{-1} \) (mg of chlorophyll\(^{-1} \)). The present communication demonstrates that the kinetics of oxidative phosphorylation catalyzed by bovine heart submitochondrial particles are consistent with negative cooperativity with respect to [ADP] and positive cooperativity in catalysis.

MATERIALS AND METHODS

The sources of chemicals used were as follows: ADP, Boehringer Mannheim; NAD and NADH, P-L Biochemicals; hexokinase, Tins, potassium succinate, and DL-\( \beta \)-hydroxybutyrate, Sigma; Demerol, Winthrop; CCCP, Calbiochem; and carrier-free \(^32\)P, ICN. Other chemicals were reagent grade.

Phosphorylating submitochondrial particles (SMP) were prepared from bovine heart mitochondria essentially according to Hansen and Smith (13). Heavy layer beef heart mitochondria were prepared from freshly isolated mitochondria according to Hatefi and Lester (14), suspended at 50-70 mg of protein/ml in 0.25 M sucrose, containing 10 mM Tris acetate, pH 7.5, 1.5 mM ATP, and 10 mM MgCl\(_2\), frozen in liquid nitrogen, and stored frozen at −70 °C for variable lengths of time up to 6 months. The frozen mitochondrial suspension was thawed at room temperature, homogenized, adjusted to a final protein concentration of 40 mg/ml with 0.25 M sucrose, 10 mM Tris acetate, pH 7.5, and supplemented with 1 mM potassium succinate, 1.5 mM ATP, 10 mM MgCl\(_2\), and 10 mM MnCl\(_2\). The suspension was subjected to 100-ml batches to sonication in a rosette cell for 1 min at 0 °C, followed by centrifugation at 10,000 rpm. The supernatant layer was carefully decanted, leaving behind the loosely packed residue, and recentrifuged in a No. 40 rotor for 45 min at 49,000 rpm. The pellet was washed once in a buffer containing 0.25 M sucrose and 10 mM Tris acetate, pH 7.5, suspended by homogenization in the same buffer at 40-60 mg of protein/ml, frozen in liquid nitrogen in small aliquots, and stored at −70 °C. There was no appreciable loss of oxidative phosphorylation activity after storage for a period of at least 6 months. Protein concentration was determined by the biuret method (15) in the presence of 1 mg of deoxycholate/ml.

Oxidative phosphorylation activity was measured at 30 °C essentially as described before (16). In a final volume of 0.6 ml, the reaction mixtures at pH 7.5 contained 0.25 M sucrose, 50 mM Tris acetate, 0.5 mM EDTA, 25 mM glucose, 5 mM MgCl\(_2\), 20 mM potassium phosphate (1-3 × 10^{-4} \text{ cmol/mol of } ^{32}\text{P}), 1-1200 \mu M \text{ADP, } 70 \mu g \text{ of hexokinase/}\text{ml, and } 50 \mu g \text{ of SMP/ml. When NADH was the respiratory substrate, the mixture was preincubated for 4 min at 30 °C, then the reaction was initiated by the addition of 0.5 mM NADH and terminated after 4 min (or as otherwise indicated) by the addition of 50 \mu l of 35% perchloric acid. In the } \beta \text{-hydroxybutyrate-driven reaction, } 30 \text{ mM DL-} \beta \text{-hydroxybutyrate acid and } 1 \text{ mM NAD were added instead of NADH. Where indicated, Demerol in an aqueous solution or CCCP in ethanol was added to the reaction mixture prior to NADH addition. When

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\(^1\) The abbreviations used are: CCCP, carbonyl cyanide m-chlorophenylhydrazone; SMP, bovine heart submitochondrial particles.
Cooperativity in ATP Synthesis

Experimental conditions

(i) There are two kinds of F1-ATPase, each having its own characteristic \( K_{m}^{DP} \) and \( V_{max} \) for ATP synthesis. (ii) The catalytic sites on F1-ATPase act independently, and in the ADP concentration range of 1-1200 \( \mu \)M exhibit the two \( K_{m} \) and \( V_{max} \) values shown in Table I. (iii) The catalytic sites on F1-ATPase exhibit cooperativity. The first possibility is the high phosphorylation activity of the SMP used in the data of Fig. 2 may have evoked in the reader’s mind, the last is the most probable because it has been established that the reverse reaction, i.e. ATP hydrolysis, catalyzed by isolated or membrane-bound F1-ATPase is marked by site-site cooperativity (3-8).

It may be important at this point to discuss a question that the data of Fig. 2 may have evoked in the reader’s mind, namely, why was this apparent cooperativity in oxidative phosphorylation never before observed? Two factors are clearly important for demonstration of this phenomenon. One factor is the high phosphorylation activity of the SMP used (note the apparent \( V_{max} \) of 2340 nmol of ATP-min\(^{-1}\)-mg\(^{-1}\) in Table I; the experimentally determined rate of ATP synthesis at 1200 \( \mu \)M ADP was 2180 nmol-min\(^{-1}\)-mg\(^{-1}\)). The other factor is the nature of the respiratory substrate and the rate of electron transfer. In oxidative phosphorylation experiments catalyzed by SMP, the respiratory substrate commonly used is either succinate or a system that generates NADH at a slow rate (e.g. \( \beta \)-hydroxybutyrate + NAD or alcohol dehydrogenase + ethanol + low levels of NAD). Substrate quantities of NADH are rarely employed, in part because the ATP yield

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**Fig. 1. Time course of ATP synthesis \(^{32P} \) esterification catalyzed by SMP respiring on NADH in the presence of various fixed concentrations of ADP. Experimental conditions were the same as described under “Materials and Methods,” except that the reactions were terminated at the time points indicated. ADP concentrations (\( \mu \)M) and the rates of oxidative phosphorylation (nmol of \(^{32P} \) esterified-min\(^{-1}\)-mg of SMP\(^{-1}\)) calculated from the slopes of the lines were, respectively: O, 400 and 1920; 

\( \triangle \), 40 and 890; 

\( \Delta \), 4 and 200; and 

A, 1 and 73.

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**TABLE I**

Apparent \( K_{m}^{DP} \) and \( V_{max} \) values calculated from the Eadie-Hofstee plots of Figs. 2 and 3

| Respiratory substrate | \( K_{m} \) | \( V_{max} \) | \( K_{m} \) | \( V_{max} \) | Total \( V_{max} \) |
|----------------------|----------|----------|----------|----------|-----------------|
| NADH                | 9.2      | 500      | 115      | 1840     | 2340            |
| Succinate           | 6.9      | 590      | 120      | 106      | 650             |
| NADH + 1 mM Demerol*| 8.8      | 415      | 105      | 986      | 1401            |
| NADH + 2 mM Demerol | 9.3      | 287      | 95       | 213      | 500             |
| \( \beta \)-Hydroxybutyrate + NAD* | 8.6      | 225      | 114      | 34       | 259             |
| NADH + 0.58 \( \mu \)M CCCP | \( \leq 17 \) | 63       | 383      | 591      | 654             |

*Kinetic data are not shown in the figures.

The kinetics of oxidative phosphorylation driven by \( \beta \)-hydroxybutyrate oxidation (Fig. 3) could also be analyzed in terms of a single straight line, but the points at the highest ADP concentrations did not fit a straight line.

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**Fig. 2.** Eadie-Hofstee plots of the kinetics of oxidative phosphorylation catalyzed by SMP respiring on NADH (●) or succinate (■). Experimental conditions were the same as described under “Materials and Methods.” $v$ is the rate of ATP synthesis in nmol·min$^{-1}$·(mg of SMP protein)$^{-1}$, and $[S]$ is ADP concentration (1 to 1200 µM). This and Fig. 3 are computer printouts, and the dots represent the curves calculated from the $K_m$ and $V_{max}$ values (see Table I), which were obtained by computer-assisted curve fitting as described elsewhere (8). The inset is a replot of the data of the succinate-driven reaction on an expanded abscissa.

**Fig. 3.** Eadie-Hofstee plots of the kinetics of oxidative phosphorylation showing the effects of respiration rate attenuation and partial uncoupling. Experimental conditions were the same as described under “Materials and Methods.” Where indicated the respiratory substrate was NADH in the presence of 2 mM Demerol (■), NADH in the presence of 0.58 µM CCCP (●), or β-hydroxybutyrate plus NAD (+). The inset shows on a compressed scale replots of the Demerol-inhibited reaction (■) and the data of Fig. 2 for the uninhibited NADH-driven reaction (●).

(P/O ratio) diminishes at high rates of respiration. However, as we have pointed out elsewhere (19), attenuation of the rate of respiration lowers the observed apparent $K_m$ values for ADP and $P_i$. This effect appears to be greater on the high $K_m$ than on the low $K_m$ values. Consequently, the contribution of the high $K_m$ component to the overall kinetics tends to become less and less discernible as the rate of respiration is further attenuated. Results demonstrating this point are shown in Fig. 3. The inset shows the Eadie-Hofstee plot of the NADH-driven data of Fig. 2 (open rectangles) and a plot of data for...
Cooperativity in ATP Synthesis

a similar experiment in which the rate of NADH oxidation was inhibited by 80% by the addition of 2 mM Demerol to the reaction mixture (solid rectangles). Comparison of the two plots shows clearly that when the rate of NADH oxidation was attenuated the contribution of the high $K_{m}^{ADP}$ component to the overall plot was diminished. The main section of Fig. 3 shows in expanded form the Demerol attenuated data of the inset (solid rectangles) next to a plot obtained with $\beta$-hydroxybutyrate + NAD as the respiratory substrate (crosses). The overall $V_{max}$ in the latter case was even less than that in the presence of NADH and 2 mM Demerol, and the data fitted a single straight line up to about 100 $\mu$M ADP and tended to deviate from linearity only at the highest ADP concentrations used. In order to obtain further support for the contention that attenuation of the rate of respiration tends to diminish the curvilinearity of the Eadie-Hofstee plots by changing the observed $K_{m}^{ADP}$ values toward lower values, another experiment was performed in which oxidative phosphorylation was partially uncoupled by the addition of 0.58 $p~$CCCP. The extent of uncoupling was so adjusted that the overall $V_{max}$ was close to that obtained in the experiment in which the rate of NADH oxidation was attenuated by the addition of 2 mM Demerol. However, as we have shown elsewhere (16, 19) (see also Ref. 20), uncoupling affects the observed $K_{m}$ values for ADP and $P_{i}$ in the opposite direction, i.e. whereas attenuation of respiration decreases these $K_{m}$ values, partial uncoupling increases them. Thus, one would expect that in the presence of partially uncoupling concentrations of CCCP, the contribution of the low $K_{m}^{ADP}$ component should be diminished in the Eadie-Hofstee plots, and the contribution of the high $K_{m}^{ADP}$ component should be exaggerated. This is precisely what was observed, as seen in Fig. 3 (solid circles).

Two additional points should be discussed. (i) At the same overall $V_{max}$ for ATP synthesis, the Eadie-Hofstee plot obtained with NADH as the respiratory substrate (in the presence of partially inhibitory concentration of either Demerol or rotenone, data not shown) was more curvilinear than that obtained with succinate as the energy source. The reason for this difference is under investigation. It may be pointed out, however, that the two experiments are not identical, because electrons emanating from NADH go through one additional coupling site (site 1) as compared to those from succinate. (ii) Except for the values obtained in the presence of CCCP, all the low $K_{m}^{ADP}$ (7–9 $\mu$M) and the high $K_{m}^{ADP}$ (95–120 $\mu$M) values obtained under various conditions appeared to be nearly the same (Table I). However, it should be emphasized that the only significant aspects of these numbers are their order of magnitude and the fact that the low and the high $K_{m}^{ADP}$ values differ by at least a factor of 10. As discussed above and in detail elsewhere (16, 19), the $K_{m}$ values measured for ADP and $P_{i}$ will depend on the degree of coupling of the SMP preparation used and the electron transfer rate obtained with various respiratory substrates. The same factors appear to affect the $K_{m}^{ADP}$ ($K_{n}^{P}$ was not investigated) in photophosphorylation (21).

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