2,4-DINITROPHENOL CAUSES A MARKED INCREASE IN THE APPARENT K_m OF P_i AND OF ADP FOR OXIDATIVE PHOSPHORYLATION

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SUMMARY: During oxidative phosphorylation by beef-heart submitochondrial particles, the presence of an uncoupler, 2,4-dinitrophenol, causes over a ten-fold increase in the apparent Michaelis constants of P_i and of ADP. These results are consistent with an energy-linked conformational change that promotes the binding of P_i and ADP in a mode competent for ATP synthesis.

INTRODUCTION

Several years ago the uncoupler insensitivity of the $P_i \rightleftharpoons HOH$ exchange catalyzed by mitochondria led to the suggestion that a prominent function of energy in oxidative phosphorylation was to promote the release of a tightly-bound ATP (1). Extension of these studies demonstrated that energy input also promotes binding of P_i and/or ADP in a mode competent for ATP synthesis (2). Additional results have led to an alternating dual-site model in which an energy-requiring conformational transition loosens ATP binding at one site and promotes competent binding of ADP and P_i at the other (3).

If energy input does serve to promote formation of a productive complex of enzyme, P_i, and ADP, the apparent Michaelis constants for P_i or ADP or both might be increased in the presence of uncouplers of oxidative phosphorylation. Somewhat unexpectedly an assessment of this possibility apparently has not been made. The purpose of this paper is to report such an assessment, and the finding that 2,4-dinitrophenol causes a marked increase in the apparent Michaelis constants for both P_i and ADP in net oxidative phosphorylation by beef-heart submitochondrial particles.

EXPERIMENTAL

Preparations and assays were performed by conventional procedures (see 1-3). For linearity of oxidative phosphorylation with time, it was necessary to incubate submitochondrial particles with succinate for a short period prior to addition of P_i and ADP. Use of a relatively high hexokinase level was important at low ADP concentrations to maintain ADP at a nearly constant level during rapid net glucose 6-phosphate formation.

For measurements of oxidative phosphorylation rates, submitochondrial particles (0.5 mg protein) were incubated for 3 min in 0.9 ml at 30° in a reaction mixture that, after dilution to 1.0 ml, would give final concentrations at pH 7.5 of 15 mM succinate, 60 mM Tris, 45 mM glucose, 4 mM $MgCl_2$ and 250 µg of yeast hexokinase. ATP synthesis was initiated by addition of varied amounts of $^{32}P_i$ or ADP at the same temperature and pH and incubation continued for 1 min. When used, 2,4-dinitrophenol was added with the P_i and ADP. Reactions were quenched with perchloric acid and formation of glucose 6-[^{32}P] phosphate was measured. Separate experiments showed that oxidative phosphorylation rates were linear under the conditions and time of the assays.

RESULTS

Measurements were made of rates of ATP synthesis with Mg^{++}-ADP held

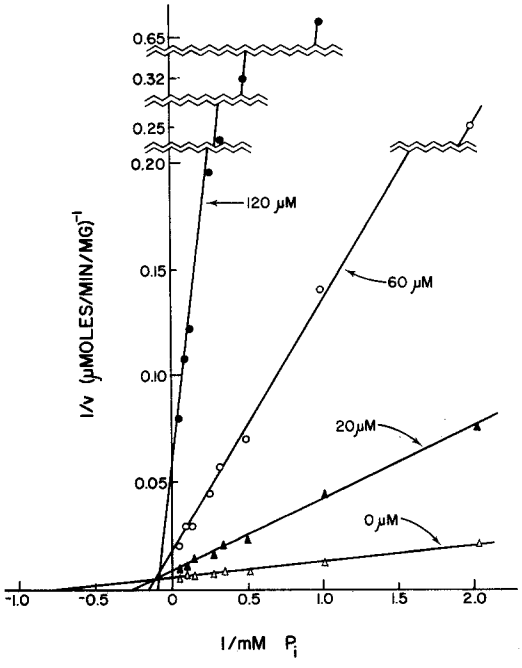

FIGURE 1

Reciprocal plots for the effect of 2,4-dinitrophenol on the rate of oxidative phosphorylation at increasing concentrations of K^+-P_i and constant 3.0 mM Mg^{++}-ADP. The 2,4-dinitrophenol concentrations are shown in the figure.

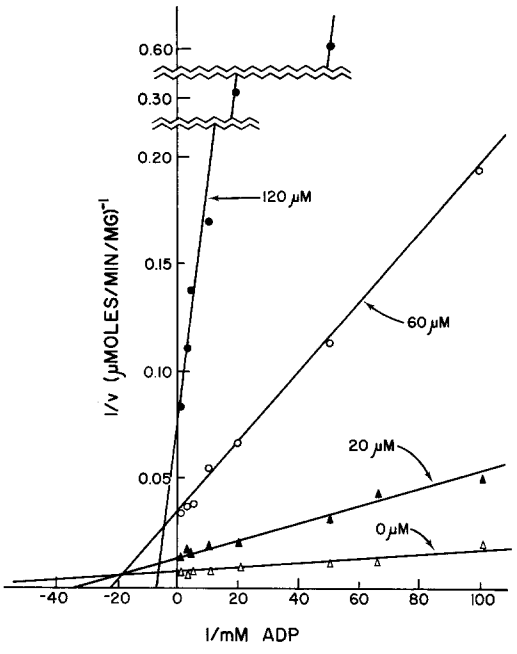

FIGURE 2

Reciprocal plots for the effect of 2,4-dinitrophenol on the rate of oxidative phosphorylation at increasing concentrations of Mg^{++}-ADP and constant 20 mM K^{+}-P_i. The 2,4-dinitrophenol concentrations are shown in the figure.

constant at 3 mM and with 0.5, 1, 2, 3, 4, 8, 10 or 20 mM K^{+}-P_i, or with K^{+}-P_i held constant at 20 mM and with 10, 15, 20, 50, 100, 200, 333 or 1000 μM Mg^{++}-ADP. 2,4-Dinitrophenol was present at 0, 30, 60 and 120 μM. With 120 μM 2,4-dinitrophenol the ATP formation rate (1 ml mM Mg^{++}-ADP and 20 mM Mg^{++}-P_i) was reduced by about 90%; this was the highest 2,4-dinitrophenol concentration compatible with satisfactorily measureable rates of oxidative phosphorylation.

Data obtained are shown as 1/v vs 1/S plots in Figs. 1 and 2. The velocity-substrate relationships for both substrates correspond to that expected for simple Michaelis behavior. The expected marked decrease in V_{max} produced by 2,4-dinitrophenol is readily apparent. The estimated Michaelis constants from the plots are given in Table 1. Also shown in Table 1 are values for the con-

TABLE 1

Estimated Values of Michaelis Constants

Concentration of 2,4-dinitrophenol	K_m Values for P_i		K_m Values for ADP	
	$\frac{1}{v}$ vs $\frac{1}{S}$	$\frac{v}{S}$ vs v	$\frac{1}{v}$ vs $\frac{1}{S}$	$\frac{v}{S}$ vs v
μM	mM	mM	μM	μM
0	1.3	1.0	13	14
20	4.0	3.9	29	25
60	6.9	7.2	48	51
120	12.0	10.5	140	140

stants as estimated from v vs v/S plots (Eadie-Hofstee plots, not shown). The results demonstrate that an about ten-fold increase in the apparent K_m values for both P_i and ADP occurs with increase in the 2,4-dinitrophenol concentration to 120 μM. Obviously the effect of the uncoupler on the apparent K_m has not reached a maximum, and if measurements were feasible at higher uncoupler concentration, an increase in K_m values considerably above ten-fold would be anticipated.

Our observed values for apparent K_m in absence of uncoupler appear to be in reasonable agreement with some of the values reported by others with heart or liver submitochondrial particles under slightly different conditions. Thus for P_i, Lee and Ernster (4) reported an apparent K_m value of 1.7 mM, Bygrave and Lehninger (5) of 6 mM, and Mitchell et al. (6) of 0.7 mM. For ADP, Hohnadel and Cooper (7) reported an apprent K_m value of 13.6 μM, Bygrave and Lehninger (5) of 300 μM, and Catterall and Pedersen (8) of 3.8 μM.

DISCUSSION

For oxidative phosphorylation a dual-site sequence has been proposed (3) in which a key step is an energy-linked transition depicted by Equation 1,

$$ATP > \underset{\bullet P_i}{\overset{\bullet ADP}{E}} \xleftrightarrow[\text{energy}]{} ATP \bullet \overset{* \; < ADP}{\underset{< P_i}{E}} \qquad (1)$$

where $ATP > E$ denotes ATP tightly-bound at one catalytic site, $ATP \bullet E$ a loosely-bound ATP, and $\overset{* \; < ADP}{\underset{< P_i}{E}}$ a conformationally-energized form with ADP and P_i bound in a mode favoring conversion to $E < ATP$ without further energy input. The energy source depicted in Equation 1 is regarded as energy transmited from oxidations by protein-protein interactions or by a transmembrane potential and/or a proton gradient. Uncouplers, by dissipating the energy source, would be expected to modify the rate constants for the single step of energy input (Equation 1) in the entire catalytic cycle.

Modification of a single step in a simplified catalytic sequence as often given for enzyme catalysis (step 1 or 2 of Equation 2) cannot give

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E \cdot S \xrightarrow{k_2} E + P \qquad (2)$$

both a decrease in V_{max} and in increase in K_m. However, as is well-recognized, a minimal reaction scheme for enzyme catalysis should include at least the number of steps depicted by Equation 3.

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E \cdot S \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E \cdot P \xrightarrow{k_3} E + P \qquad (3)$$

For this sequence,

$$V_{max} = \frac{k_2 k_3 E_t}{k_2 + k_{-2} + k_3}$$

and

$$K_m = \frac{k_{-1} k_{-2} + k_{-1} k_3 + k_2 k_3}{k_1 (k_2 + k_{-2} + k_3)}$$

From these relationships it follows that a decrease in k_2 and a corresponding increase in k_{-2} must decrease V_{max} and could readily increase K_m. For example, an increase in k_{-2} together with an equal decrease in k_2 would increase K_m if $k_{-1} > k_3$. The increase in K_m would not be accompanied by an increase in the dissociation constant for E·S, K_d, given by k_{-1}/k_1.

Such considerations are relevant to the proposed dual-site sequence for oxidative phosphorylation (3). An uncoupler modifying the energy input step might readily increase the apparent K_m without necessarily changing the dissociation constant for P_i or ADP. Presence of an uncoupler would, however, be expected to decrease the steady-state concentration of $\overset{*}{E}\begin{matrix}< ADP \\ < P_i\end{matrix}$ in the phosphorylation sequence. Correspondingly, energy input could decrease the apparent K_m values for P_i and ADP and increase the steady-state concentration of $\overset{*}{E}\begin{matrix}< ADP \\ < P_i\end{matrix}$ without necessarily changing the K_d values. This explanation gives additional understanding to why we state that energy input promotes the binding of P_i and ADP in a mode competent for the formation of bound ATP (2,3).

Our results showing that energy-linked conformational transitions may change apparent Michaelis constants for substrates are in harmony with the earlier findings of Rydström et al. for the energy-linked transhydrogenase of submitochondrial particles (9).

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