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Substrate binding affinity changes in mitochondrial energy-linked reactions

(ATP synthesis/ATP–P_i exchange/reverse electron transfer/nicotinamide nucleotide transhydrogenation/enzyme–substrate affinity)

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ABSTRACT **The effects of uncouplers and valinomycin plus nigericin (in the presence of K^+) were studied on the apparent K_m for substrates and apparent V_{max} of the following energy-linked reactions catalyzed by submitochondrial particles: oxidative phosphorylation, NTP–$^{33}P_i$ exchange, ATP-driven electron transfer from succinate to NAD, and respiration-driven transhydrogenation from NADH to 3-acetylpyridine adenine dinucleotide phosphate. In all cases, partially uncoupling (up to 90%) concentrations of uncouplers or valinomycin plus nigericin were found to decrease apparent V_{max} and to increase apparent K_m. Results plotted as ln (V_{max}/K_m) versus the concentration of uncouplers or ionophores showed a linear decrease of the former as a function of increasing perturbant concentration (i.e., decreasing free energy). Because V_{max}/K_m may be considered as a measure of the apparent first-order rate constant for enzyme–substrate interaction and reflects the affinity between enzyme and substrate to form a complex, the results are consistent with the interpretation that membrane energization leads to a change in enzyme conformation with the resultant increase in enzyme–substrate affinity and facilitation of the reaction rate under consideration. The significance of these findings with respect to the mechanism of action of the energy-transducing systems studied is discussed.**

Our previous studies of energy-linked transhydrogenation from NADH to two NADP analogs as catalyzed by submitochondrial particles (SMP) showed that membrane energization resulted in a 40-fold increase in V_{max}/K_m (decrease in apparent K_m and increase in apparent V_{max}) for both the reduced and oxidized substrates (1). The V_{max}/K_m increases were pH dependent, and the highest values were obtained at pH 7.5, the optimal pH for mitochondrial energy-linked processes. These results indicated that, in NADH → NADP transhydrogenation, enzyme–substrate affinity as reflected by V_{max}/K_m (2) is modulated by the energized state of SMP and suggested that the basis of this energy-linked modulation might be a $\Delta\mu_{H^+}$-induced enzyme conformation change resulting in an increased rate of enzyme–substrate interaction and a consequent facilitation of hydride ion transfer from NADH to NADP (1). In other studies, thermodynamic results consistent with the possible $\Delta\mu_{H^+}$-induced conformation change of the transhydrogenase enzyme were also presented (3).

In the present paper, we report on the modulation of V_{max} and K_m by uncouplers and by valinomycin plus nigericin for four energy-linked reactions of mitochondria. The reactions studied were (*i*) ATP synthesis, (*ii*) NTP–$^{33}P_i$ exchange, (*iii*) nicotinamide nucleotide transhydrogenation, and (*iv*) ATP-driven electron transfer from succinate (succinate/fumarate $E_{m,7}$ = +30 mV) to NAD (NADH/NAD $E_{m,7}$ = −315 mV).

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METHODS AND MATERIALS

SMP was prepared from bovine heart mitochondria essentially according to Löw and Vallin (4) as described elsewhere (1). Protein concentration was determined by the biuret method (5) in the presence of 1% potassium deoxycholate. The assays for oxidative phosphorylation, NTP–$^{33}P_i$ exchange, ATP-driven electron transfer from succinate to NAD, and energy-linked transhydrogenation were carried out as follows.

Oxidative Phosphorylation. To 0.3 ml of a solution containing 100 mM Tris acetate, 500 mM sucrose, 1 mM EDTA, 50 mM glucose, and 0.5–40 mM potassium phosphate at pH 7.5 and 30°C were added 1–2 × 10^6 cpm of $^{33}P_i$, 42 μg of hexokinase, 1.8–2.55 μmol of $MgCl_2$, and water to 0.513 ml. Then, 60 μg of SMP (3 μl) and 12 μmol of sodium succinate (24 μl) were added, and the mixture was incubated at 30°C for 3 min. Uncouplers and ionophores were added as indicated. The reaction was started by addition of 12–300 nmol of ADP (60 μl) and terminated after 3 min by the addition of 60 μl of 35% perchloric acid. Precipitated protein was removed by centrifugation for 10 min at top speed in a clinical centrifuge, and 0.5 ml of the supernatant was used for estimation of esterified ^{33}P essentially according to Pullman (6) as described by Stiggall *et al.* (7).

NTP–$^{33}P_i$ Exchange. NTP–$^{33}P_i$ exchange activity was measured essentially according to Stiggall *et al.* (7) with one modification. Different preparations of SMP vary in their ATPase and ATP–P_i exchange activities. Aging at −70°C increases ATPase activity and decreases ATP–P_i exchange. The variable low rates of exchange and the variable high rates of ATP hydrolysis in SMP are due to the proton leakiness of SMP vesicles, and we have found that the presence of an oxidizable substrate such as succinate in the NTP–P_i exchange assay medium increases the exchange rate severalfold and minimizes variations of the exchange rate from one experiment to another. The $^{33}P_i$ exchange rates, especially with GTP, ITP, and UTP, were found to be faster than NTP synthesis by oxidative phosphorylation under the same conditions (i.e., among other things, low NDP concentration), and the extent of ATP hydrolysis during the ATP–P_i exchange reaction time was found to be the same in the absence and presence of succinate. These results, which will be published elsewhere, indicated that NTP–$^{33}P_i$ exchange in the presence of succinate was a more reliable measure of the exchange activity of SMP and was not complicated greatly by *de novo* NTP synthesis.

Accordingly, the NTP–$^{33}P_i$ exchange assay medium at 30°C contained, in a final volume of 0.6 ml, 300 mM sucrose, 25 mM Tris acetate at pH 7.5, 1.8 mg of bovine serum albumin, 20 mM

Abbreviations: SMP, phosphorylating beef-heart submitochondrial particles; DNP, 2,4-dinitrophenol; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; S-13, 5-chloro-3-*t*-butyl-2′-chloro-4′-nitrosalicylanilide; AcPyADP, 3-acetylpyridine adenine dinucleotide phosphate.

sodium succinate, $MgSO_4$ at 1.25 times the molar concentration of the NTP present, 60 μg of SMP, and 1–2 × 10^6 cpm of $^{33}P_i$ (0.5–1 mM) plus potassium phosphate (pH 7.5) to the concentrations indicated. The reaction was started by the addition of NTP as indicated and terminated 6 min later with 60 μl of 35% perchloric acid. Esterified ^{33}P was determined as above.

Electron Transfer from Succinate to NAD. The 1-ml cuvette at 38°C contained 250 mM sucrose, 50 mM Tris sulfate (pH 7.5), 6 mM $MgCl_2$, 0.1% bovine serum albumin, 5 mM sodium succinate, 4.5 mM ATP, 10 mM KCN, and 0.2 mg of SMP. The reaction was started by addition of NAD as indicated and was followed at 340 nm. The millimolar extinction coefficient of NADH used for calculation of activities was 6.22. The uncoupler S-13 was preincubated at the concentrations indicated with SMP (10 mg/ml) at 0°C and was added to the assay medium together with SMP.

Energy-Linked Transhydrogenation. The reaction studied was the reduction of 3-acetylpyridine adenine dinucleotide phosphate (AcPyADP) by NADH as catalyzed by SMP and energized by succinate oxidation. The reaction mixture, in a final volume of 1.0 ml at 37°C, contained 250 mM sucrose, 100 mM Hepes adjusted to pH 7.5 with KOH, 10 μg of rutamycin, 25 μM rotenone, 40 μM NADH, 173 and 72 μg of SMP in the experiments of Figs. 3 and 4, respectively, and AcPyADP as indicated. Ionophores or S-13 was added to the assay mixture after the addition of SMP, and the reaction was started by addition of 10 mM sodium succinate. The reduction of AcPyADP was followed in the Aminco DW-2a dual-wavelength spectrophotometer by the ΔA between 375 and 425 nm. Rates were calculated by using an absorbance difference value of 6.38 $mM^{-1} \cdot cm^{-1}$ for AcPyADPH minus NADH (8).

Adenine nucleotides were from Boehringer, nicotinamide nucleotides, UTP, GTP, and ITP were from P-L Biochemicals, S-13 was from Monsanto, and carbonylcyanide *m*-chlorophenylhydrazone (CCCP) and valinomycin were from Calbiochem. $^{33}P_i$ (carrier free) was obtained from New England Nuclear and was recrystallized as before (7). Rutamycin and nigericin were gifts from Robert L. Hamill (Eli Lilly). Other chemicals were reagent grade.

RESULTS

Effects of Uncouplers. Figs. 1 and 2 show the effect of partially inhibiting (up to 90%) concentrations of the uncoupler CCCP on the apparent K_m for ADP and P_i and apparent V_{max} in oxidative phosphorylation as catalyzed by SMP and driven by succinate oxidation. The *Insets* in Figs. 1 and 2 show the changes in ln (V_{max}/K_m) as a function of CCCP concentration. Essentially similar results were obtained with the uncoupler 2,4-dinitrophenol (DNP) (see also ref. 10 for the effect of DNP on the apparent K_m of ADP and P_i in oxidative phosphorylation).* In addition, examples given in Fig. 3 show that uncouplers had a similar effect on V_{max}/K_m in NTP–$^{33}P_i$ exchange, ATP-driven electron transfer from succinate to NAD, and respiration-driven (succinate oxidation) transhydrogenation from NADH to 3-acetylpyridine adenine dinucleotide phosphate (AcPyADP). In Fig. 3 (and in Fig. 4 below) the maximal degree of inhibition achieved in different experiments varied between 65% and 82%. The reason for not going to higher degrees of inhibition was that, at higher uncoupler concentrations, the reaction rates became too low to measure accurately.

* We prefer to use CCCP and S-13 as uncouplers because DNP reacts with F_1-ATPase (9) and therefore would complicate the interpretation of results, especially in regard to the K_m changes of ATP, ADP, and P_i as a function of DNP concentration.

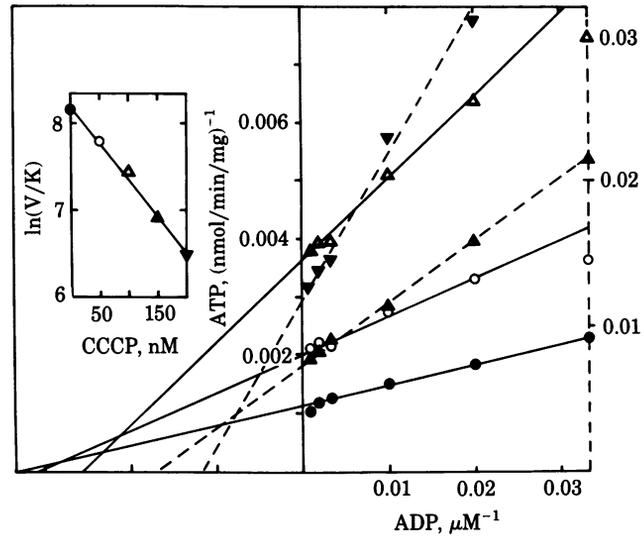

FIG. 1. Double-reciprocal plots showing the effect of partially uncoupling concentrations of CCCP on the rate of oxidative phosphorylation and apparent K_m for ADP. Dashed lines refer to the expanded, dashed ordinate scale. (*Inset*) Plot of ln (V_{max}/K_m) versus the CCCP concentration used (●, 0 nM; ○, 50; △, 100; ▲, 150; ▼, 200).

Effect of Valinomycin Plus Nigericin. Valinomycin plus nigericin in the presence of K^+ uncouples mitochondrial energy-linked reactions (11). These ionophores exhibited an effect similar to that of uncouplers on the V_{max}/K_m of the above energy-linked reactions. At the concentrations used, each ionophore, in the absence of the second ionophore, caused little or no inhibition. The experiments were carried out in two ways: (*a*) in the presence of a constant amount of valinomycin plus increasing concentrations of nigericin, and (*b*) in the presence of a constant amount of nigericin plus increasing concentrations of valinomycin. In other words, in the first case the driving force was first converted to ΔpH, then nigericin was added to suppress ΔpH in a stepwise manner. In the second case the driving force was $\Delta\psi$, which was suppressed in a stepwise manner by addition of increasing amounts of valinomycin. Fig. 4 shows examples of both types of experiments as applied to oxidative phosphoryl-

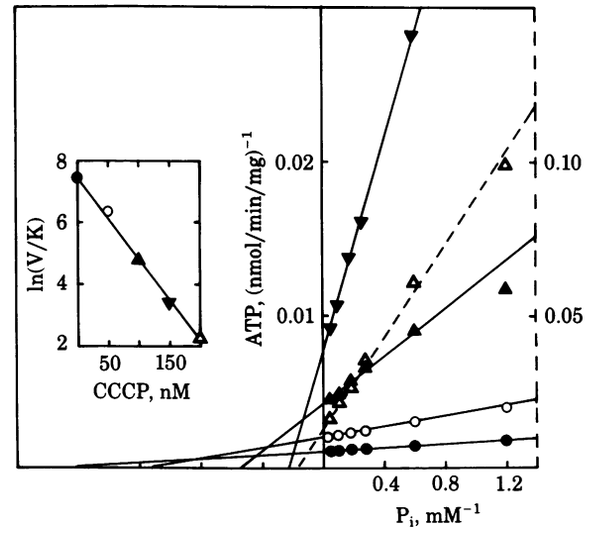

FIG. 2. Double-reciprocal plots showing the effect of partially uncoupling concentrations of CCCP on the rate of oxidative phosphorylation and apparent K_m for P_i. Dashed line refers to the expanded, dashed ordinate scale. (*Inset*) Plot of ln (V_{max}/K_m) versus the CCCP concentrations used (●, 0 nM; ○, 50; ▲, 100; ▼, 150, and △, 200).

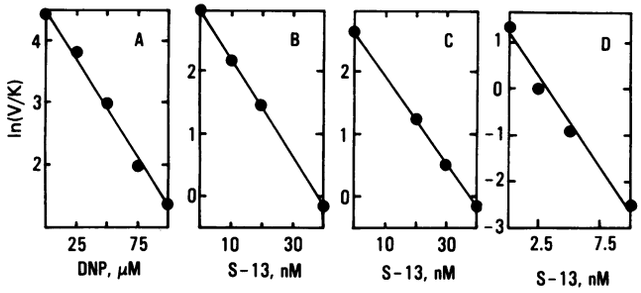

FIG. 3. Plots of ln (V_{max}/K_m) versus partially uncoupling concentrations of DNP and S-13 as shown for GTP–$^{33}P_i$ exchange (*A*), ITP–$^{33}P_i$ exchange (*B*), ATP-driven succinate reduction of NAD (*C*), and transhydrogenation from NADH to AcPyADP (*D*). The variable substrates were GTP (0.9–4.2 mM) in *A*, P_i (1.3–21.5 mM) in *B*, NAD (0.02–0.2 mM) in *C*, and AcPyADP (5–200 μM) in *D*. All apparent K_m and V_{max} values were obtained from double-reciprocal plots as in Figs. 1 and 2.

ation, ATP–$^{33}P_i$ exchange, and transhydrogenation. Uncoupling by the combined actions of valinomycin plus nigericin in the presence of K^+ also resulted in a decrease in V/K (decrease in apparent V_{max} and increase in apparent K_m).

Because uncouplers and valinomycin plus nigericin are known to decrease the magnitude of $\Delta\mu_{H^+}$, the results of Figs. 1–4 suggest that suppression of $\Delta\mu_{H^+}$ has a direct effect on the apparent rate of enzyme–substrate interaction (as expressed by V_{max}/K_m) in the four energy-requiring reactions tested. It is also interesting that, in all the cases examined, the decrease in ln (V_{max}/K_m) was essentially a linear function of increasing perturbant concentration, at least in the ranges used.

DISCUSSION

It has been shown in four energy-linked reactions catalyzed by submitochondrial particles—oxidative phosphorylation, NTP–$^{33}P_i$ exchange, ATP-driven electron transfer from succinate to NAD, and transhydrogenation from NADH to AcPyADP—that partial uncoupling (up to 90%) by addition of uncouplers or valinomycin plus nigericin (in the presence of K^+) results in a decrease in apparent V_{max} and an increase in apparent K_m for the appropriate substrates. Data were plotted as ln (V_{max}/K_m) versus uncoupler concentration (or variable ionophore concentration), and it was shown that in all cases ln (V_{max}/K_m) decreased linearly with increasing perturbant concentration, at least in the ranges examined.† On the assumption that in the systems studied V_{max}/K_m may be considered to be a measure of the apparent first-order rate constant for enzyme–substrate interaction, and hence an index of the affinity between enzyme and substrate (2), a possible interpretation of the above results is that membrane energization changes the conformation of the respective enzymes (i.e., F_1-ATPase, NADH dehydrogenase, and transhydrogenase), resulting in increased enzyme–substrate affinity and facilitation of the energy-linked reaction rate. For oxidative phosphorylation, a minimum set of reactions may be written as shown in Fig. 5, where E_1 and E_2 are the two conformations of the ATPase enzyme under nonenergized and energized conditions of the system, respectively.

Fig. 5 shows that the substrates ADP and P_i interact with E_1 in a freely reversible manner. Conversion of E_1 to E_2 under the influence of $\Delta\mu_{H^+}$ [we assume that this occurs by interaction of H_c^+ with E_1 to form H-E_2, or more precisely $(H)_n$-E_2 in which n is the number of protons taken up per mole of the enzyme] increases the affinity between H-E_2 and ADP and P_i. This altered state of enzyme–substrate interaction leads to the formation of ATP "tightly" bound to H-E_2. Then, H-E_2·ATP loses protons to the matrix side and is converted to E_1°ATP in which ATP is loosely bound and is in equilibrium with medium ATP. The forces involved are (*a*) [H_c^+] which converts E_1 to E_2 and thereby causes the important change in the mode of binding of ADP and P_i to the enzyme and the consequent synthesis of tightly bound ATP; and (*b*) the membrane potential which drives H^+ translocation from the cytosolic to the matrix side of the inner membrane and displaces the equilibrium of the coupled process toward ATP synthesis.

The above formulation is analogous to our proposed mechanism for energy-linked nicotinamide nucleotide transhydrogenation (1). It is also in agreement with the following.

1. F_1-ATPase in submitochondrial particles and chloroplasts undergoes energy-induced conformation change (9, 13–18).

2. The transhydrogenase reaction involves unrecoverable energy consumption (i.e., entropy increase) suggestive of conformation change (3).‡

3. Iron-sulfur centers N-1a and N-2 in the coupling site I region of the mitochondrial respiratory chain undergo apparent E_m change in response to phosphate potential (20, 21). Similar apparent E_m changes have been reported also for cytochromes b_{566} and a_3, which are associated with coupling sites II and III, respectively, of the respiratory chain (22, 23). These apparent E_m changes might be indicative of $\Delta\mu_{H^+}$-induced conformation changes of the respective respiratory chain enzyme complexes. In agreement with this interpretation are the following observations, which suggest that these energy transducing enzyme complexes (or components thereof) are capable of conformation change. (*a*) Respiratory chain complexes I and III are structurally more stable when reduced (24). (*b*) In submitochondrial particles, cytochrome b_{566} is reducible by substrates only when the particles are energized or treated with antimycin A (22, 25, 26). Antimycin A also increases the structural stability of complex III (27). Thus, it is possible that conditions that bring about substrate-reducibility of cytochrome b_{566} effect a conformation change in the enzyme complex. (*c*) Mild perturbations of complex III structure by low concentrations of detergents change the absorption spectrum of cytochrome b_{566} to that of cytochrome b_{562} (28), indicating that the heme environment in cytochrome b_{566} can register subtle structural perturbations of the enzyme complex. (*d*) Detachment of NADH dehydrogenase from complex I results in an increase in K_m for NADH and decreases in k_1 and V_{max} (29–31). These are the very parameters that are altered during energization and deenergization of the system.

4. The important results of Boyer and coworkers (32–37) regarding: (*a*) uncoupler-insensitive ("intermediate") and uncoupler-sensitive ("medium") P_i–H_2O oxygen exchange (in our scheme, the two forms of the enzyme-bound P_i allowing for such exchanges could be E_1°P_i and E_2·P_i respectively); (*b*) energy

† We also examined plots of ln V_{max} or ln ($1/K_m$) versus perturbant concentration, but the results were not consistent. In some cases plots of ln V_{max}, but not ln ($1/K_m$), versus perturbant concentration were nearly linear. In other cases the opposite was true or neither plot gave a linear relationship. At any rate, we do not wish to emphasize the linearity of the plots shown and, depending on the degree of proton leakiness of the SMP preparations, it is possible that, as uncoupling approaches 100%, plots of ln (V_{max}/K_m) versus perturbant concentration might deviate from linearity. The point we wish to emphasize, however, is that, in all the cases studied, V_{max}/K_m decreased appreciably as the degree of uncoupling was increased.

‡ Our proposed mechanism also agrees with the effect of membrane energization on the rate constants of the transhydrogenation reaction as reported by others (19). However, these workers assumed an ordered mechanism while a random mechanism is much more likely (1).

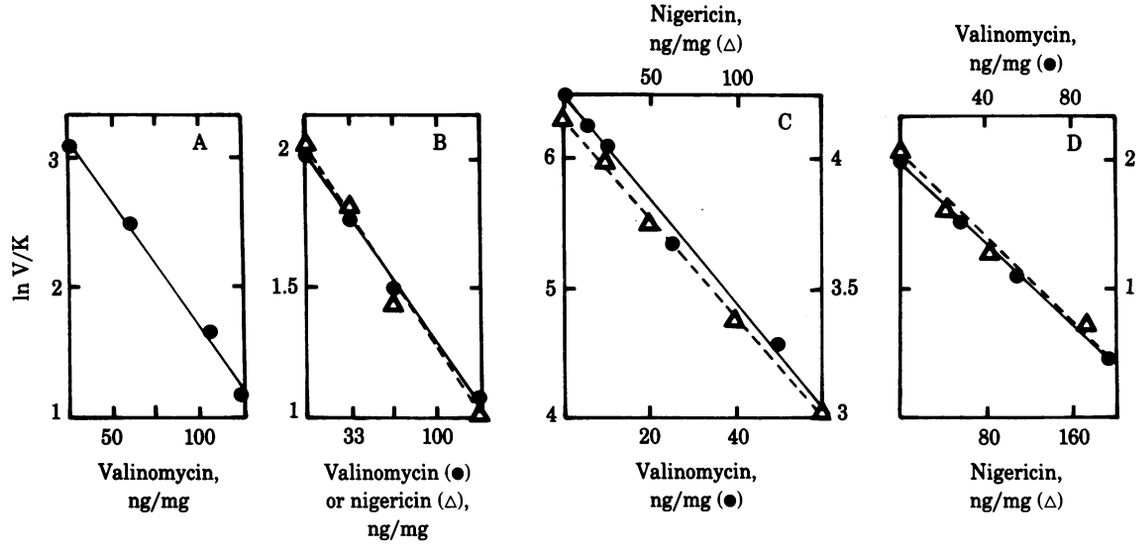

FIG. 4. Plots of ln (V_{max}/K_m) versus partially uncoupling concentrations of valinomycin plus nigericin as shown for oxidative phosphorylation with ADP (30–1000 μM) as the variable substrate (*A*), oxidative phosphorylation with P_i (0.5–20 mM) as the variable substrate (*B*), ATP–$^{33}P_i$ exchange with ATP (0.4–2.0 mM) as the variable substrate (bottom abscissa and left ordinate) and with P_i (2.5–20 mM) as the variable substrate (top abscissa and right ordinate) (*C*), and NADH → AcPyADP transhydrogenation with AcPyADP (5–100 μM) as the variable substrate (*D*). K^+ concentration varied from 33 to 50 mM. In *A*, the concentration of nigericin was 0.5 μg/mg of protein; that of valinomycin was as shown. In *B*, the concentration of valinomycin was 1.3 μg/mg of protein in the experiments marked by ●, and nigericin was as shown; the concentration of nigericin was 0.5 μg/mg of protein in the experiments marked △, and valinomycin was as shown. In *C*, the concentration of the fixed ionophore (valinomycin in △, and nigericin in ●) was 0.5 μg/mg of protein. In *D*, the concentration of the fixed ionophores was, per mg of protein, 0.56 μg of nigericin in △ and 1.25 μg of valinomycin in ●. All apparent K_m and V_{max} values were obtained from double-reciprocal plots as in Figs. 1 and 2.

requirement for substrate binding ($E_{1\circ ADP}^{\circ Pi} \xrightarrow{\text{energy}} E_{2\cdot ADP}^{\cdot Pi}$ in our scheme); and (*c*) committed phosphate ($E_2{\cdot}P_i$ in our scheme).

5. The results of Webb *et al.* (38) and Bossard *et al.* (12) suggesting that ATP hydrolysis and synthesis do not involve a phosphorylated enzyme intermediate.

6. The theoretical considerations of Jencks (39) regarding the utilization of the intrinsic binding energy between enzyme and substrate at different states of the system to facilitate a coupled vectorial process.

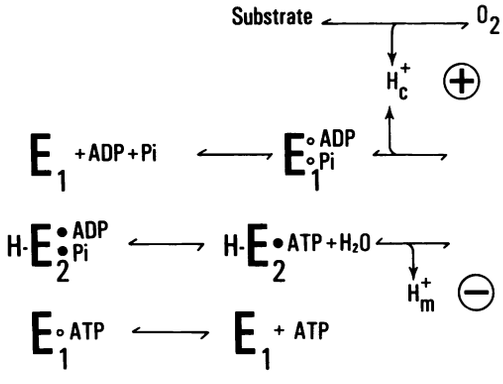

FIG. 5. Hypothetical scheme showing a minimal set of reactions for oxidative phosphorylation. E_1 and E_2, two forms of F_1-ATPase under nonenergized and energized states of the system, respectively; H_c^+ and H_m^+ chemosmotic protons on the cytosolic and matrix sides of the mitochondrial inner membrane, respectively; ⊕ and ⊖, membrane polarity under energized conditions; ● and ○, show changes in enzyme–substrate affinity. H-E_2 is written for simplicity; a more correct designation might be $(H)_n$-E_2 in which *n* is the number of protons bound per mol of ATPase. A variant of the first reaction in the scheme would be first the conversion of E_1 to H-E_2 and then the interaction of ADP + P_i with H-E_2 to form $H\text{-}E_{2\cdot Pi}^{\cdot ADP}$. Participation of Mg^{2+} has not been shown for simplicity. A possible role of Mg^{2+} might be as suggested by the studies of Bossard *et al.* (12).

It might also be of interest to add that a change in kinetic constants effected by the degree of membrane energization is not peculiar to the mitochondrial energy-linked reactions. Such changes have been reported also in the case of photophosphorylation (40) and bacterial metabolite transport (41, 42). Indeed, it is possible that the principal features of the mechanism described in Fig. 5 might be applicable to many coupled vectorial reactions, including various cation-linked ATPases.

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