Effects of Oligomycin on the Partial Reactions of the Sodium Plus Potassium-stimulated Adenosine Triphosphatase*

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The effects on phosphoenzyme (E-P) formation of ligands which activate Electrophorus (Na,K)-ATPase were investigated in the presence of oligomycin. When the enzyme was allowed to bind oligomycin in the presence of NaCl and MgCl₂, subsequent addition of ATP plus KCl produced a monoexponential time course of E-P formation with a rate of 50 s⁻¹, similar to the rate obtained in the uninhibited enzyme phosphorylated by ATP in the absence of KCl. Phosphatase under these conditions was slow and showed no initial burst phase, consistent with the inhibitory effect oligomycin had on the E₁-P to E₂-P conformational transition. Addition to KCl to a preincubation medium containing oligomycin, NaCl, and MgCl₂ had no further effect on E-P formation. However, equilibration with oligomycin, KCl, and MgCl₂ prior to the addition of NaCl plus ATP gave a much slower rate of E-P formation (5 s⁻¹) and resulted in an initial rapid release of Pᵢ, similar to that found in the uninhibited enzyme. The slow increase in E-P level observed after incubation with oligomycin, KCl, and MgCl₂ may be due to secondary formation of an inhibition complex following rapid binding of oligomycin. In contrast to the monophasic behavior which resulted from pre-exposure to NaCl or KCl, preincubation with oligomycin in the presence of MgCl₂ plus Tris or Tris alone gave a biphasic pattern of E-P formation in which about 50% of the intermediate accumulated at a rate of 56 s⁻¹ and the remainder at a rate of 5 s⁻¹. In addition, the Pᵢ burst amplitude was reduced, indicating partial inhibition of the enzyme. These results suggest that in the absence of Na⁺ and K⁺ only half of the enzyme is inhibited by oligomycin while the remainder undergoes inhibition subsequent to initiation of phosphorylation. Since the oligomycin concentration was saturating, the partial inhibition reflected in the biphasic pattern of E-P formation may be due to half-of-the-sites reactivity in which only half of the subunits bind oligomycin in the absence of monovalent cations.

The (Na,K)-ATPase, which actively transports sodium out of, and potassium into, a wide variety of cell types, operates through a cycle of phosphorylation and dephosphorylation consisting of four distinct enzymatic states: E₁ → E₁-P → E₅-P → E₅ (1–3).

Oligomycin, an inhibitor of the (Na,K)-ATPase, is thought to act by stabilizing both E₁ and E₅-P conformations (1, 4–7). Oligomycin has been shown to render the phosphorylated (Na,K)-ATPase insensitive to K⁺, which otherwise catalyzes its hydrolysis (1). This effect is consistent with an action of oligomycin to prevent the transformation of E₁-P to E₅-P which normally precedes hydrolysis.

Because oligomycin inhibits the phosphoenzyme conformational transition, it appeared to be a useful tool for studying the effects on phosphoenzyme formation of ligands which affect reactions occurring in the later portion of the ATPase cycle. In this paper, we report transient state studies on the (Na,K)-ATPase in the presence of oligomycin in which we examined the behavior of the phosphorylation reaction using the acid quench technique. By measuring the rate at which oligomycin interacts with the enzyme under a variety of ionic conditions, we have been able to show that the presence of Na⁺ in the reaction mixture greatly enhances the effects of oligomycin on the enzyme, suggesting that a state or states intermediate to weakly reacting E₅-Na and strongly reacting E₁-Na exist when neither monovalent cation is present.

MATERIALS AND METHODS

Microsomal (Na,K)-ATPase was prepared from the eel electric organ as previously described (8). Aliquots of the enzyme were frozen in liquid nitrogen and stored at –80 °C.

Because of the limited solubility of oligomycin in water, the drug was added to electroplax (Na,K)-ATPase microsomes by using one of the following methods: 1) oligomycin in 95% ethanol was added directly to a stirred suspension of the membranes; or 2) oligomycin in ethanol was air-dried onto a beaker into which the membranes were later added. In experiments where Pᵢ was measured, the latter method was used to avoid interference by ethanol with the extraction of the unhydrolyzed nucleotide by activated charcoal. The time required to inhibit the enzyme depended on which ligands were present in the reaction medium. In the absence of Na⁺, 2–5 min were required to allow the drug to equilibrate with the enzyme while in the presence of Na⁺ 1 min of equilibration time was sufficient (see "Results"). Ethanol, added to the enzyme with oligomycin, produced little or no change in the kinetics of the (Na,K)-ATPase phosphorylation reaction. Oligomycin, containing a mixture of the A, B, and C forms, was obtained from Sigma.

Rapid mixing experiments were carried out using the quench-flow device described by Froehlich et al. (9). In all of the experiments, 3 mM MgCl₂, 0.1 mM EDTA, and 50 mM Tris-HCl, pH 7.4, were present in the enzyme and substrate solutions. The enzyme protein concentration ranged from 0.3 to 1.2 mg/ml and was constant for each experiment. The reaction was terminated by the addition of a solution containing 9% perchloric acid and 6 mM phosphate. The methods for

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measuring the formation of the acid-stable phosphoenzyme and inorganic phosphate have been previously described (10). All of the experiments were performed at 21 °C.

Kinetic data were analyzed by computer simulation using MLAB (11). The procedure for analyzing the kinetics of the conformational transition involving the dephosphoenzyme is described in detail elsewhere (12).

**RESULTS**

Effects of Equilibration of Oligomycin with (Na,K)-ATPase in the Presence of Na⁺ or K⁺ on the Rate of Appearance of K⁺-Insensitive Phosphoenzyme—The rate of appearance of K⁺-insensitive E-P can be detected in a rapid quenching experiment in which the enzyme is acid-quenched at various times after the simultaneous addition of [γ-32P]ATP, Na⁺, and K⁺. By including K⁺ in the reaction medium, phosphoenzyme produced by the uninhibited enzyme will undergo rapid dephosphorylation leaving behind phosphoenzyme that is due primarily to the inhibited enzyme. The effects of Na⁺ and K⁺ on the time course of appearance of K⁺-insensitive E-P subsequent to equilibration of (Na,K)-ATPase with oligomycin are shown in Fig. 1.

In all three cases 3 mM MgCl₂ was present with oligomycin, enzyme, and buffer for approximately 1 h before rapid mixing with the phosphorylation solution. The addition of Na⁺ or K⁺ to the equilibration solution had opposite effects on the rate of appearance of K⁺-insensitive E-P. The formation of K⁺-insensitive E-P when Na⁺ is present during equilibration with oligomycin is described by a single rate constant of 4 - 5 s⁻¹. This rate is similar to the rate of phosphorylation of the enzyme in the absence of K⁺ and oligomycin under otherwise similar conditions (Fig. 2A and Ref. 10). Addition of 20 mM KCl to a medium already containing oligomycin and 100 mM NaCl had no effect on the rate of phosphorylation as shown in Fig. 2B. However, when the enzyme was incubated with K⁺ and oligomycin and later mixed with ATP plus Na⁺, the rate of formation of the K⁺-insensitive E-P was much slower, about 5 s⁻¹ (Fig. 1).

The time course of formation of K⁺-insensitive E-P subsequent to equilibration of (Na,K)-ATPase with oligomycin and Mg²⁺ and in the absence of Na⁺ and K⁺ is biphasic; about half of the phosphoenzyme appears at the rapid rate of 56 s⁻¹ and the remainder (42%) appears at about 5 s⁻¹. An identical result was obtained with an enzyme medium that contained only Tris buffer in addition to oligomycin (not shown), indicating that Mg²⁺ is not required for the observed biphasicity.

Two points should be noted with respect to this experiment. First, oligomycin equilibrates with (Na,K)-ATPase in a minute or so (see below) whereas the actual time of equilibration in this experiment was 1 h. It is evident that the different kinetics cannot reflect different rates of oligomycin binding prior to initiation of phosphorylation. Second, during the phosphorylation phase of the experiments the ligand concentrations were identical in each case. Thus, the different kinetics are not attributable to any difference in the environment of the (Na,K)-ATPase during the phosphorylation phase. The explanation must be sought in terms of some differences in the ligand-induced states of the (Na,K)-ATPase which exist after 1 h of equilibration with oligomycin and which transform at differing rates to a common state under "turnover" conditions.

From these considerations it is difficult to escape the conclusion that, in the absence of either Na⁺ or K⁺, the (Na,K)-ATPase must exist in a state different from those produced...
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by liganding with either Na⁺ or K⁺, respectively. This state is characterized by the inaccessibility of about half of the phosphorylation sites to the action of oligomycin over a period of about 1 h.

To investigate these observations further, it seemed useful to consider hypothetical explanations. A dimer hypothesis permits one to propose that Na⁺ and K⁺ binding produce the E₁/E₁ and E₂/E₂ states, respectively, whereas in their absence an E₁/E₂ state exists. The 5 s⁻¹ constant would reflect the rate of phosphorylation of E₁, which exists at the time of addition of [γ⁻³²P]ATP, whereas the 5 s⁻¹ rate would be determined by the rate of formation of additional E₁ stabilized by complexes with oligomycin subsequent to initiation of phosphorylation.

Many kinetic properties which suggest oligomeric interactions can be explained by alternative mechanisms involving only monomeric forms (13). One such alternative to explain oligomycin interactions assumes that an initial complex, E₁(o1igo), is formed which retains full catalytic activity. A further conformational transition occurs to produce the inactive complex, E'₁ (oligo). To account for the behavior in Fig. 1, the equilibrium between these two forms would be about 1 in the absence of Na⁺, whereas Na⁺ binding would stabilize the inactive form.

As noted above, accumulation of phosphoenzyme after incubation with K⁺ and oligomycin is much slower (5 s⁻¹) than the rate observed under similar conditions except for the absence of oligomycin (12). Two possible explanations are (a) that oligomycin actually binds to E₁ and slows the transition to E₁ that must precede phosphorylation, or (b) oligomycin only binds to E₁ conformers and the slow accumulation of K⁺-insensitive E-P results from slow formation of the inhibition complex between oligomycin and E₁, conformers that become available when enzyme turnover is initiated. To distinguish between these possibilities we incubated the enzyme under different liganding conditions and measured the rate of phosphate release after initiation of turnover (Fig. 3). With 100 mM Na⁺ present initially with oligomycin almost no Pi production occurred. With 100 mM Na⁺ and 20 mM K⁺ present, phosphate production occurred after a lag phase. With 20 mM Na⁺ and 100 mM K⁺ a large Pi burst was observed, demonstrating that K⁺ prevents formation of the inhibited complex. Since Pi release is faster in the presence of high K⁺, oligomycin evidently does not slow the conversion of E₂ to E₁ as postulated above. These effects are qualitatively consistent with the earlier evidence that oligomycin inhibition is promoted by Na⁺ and antagonized by K⁺ (14, 15).

When Na⁺ and K⁺ are both absent during equilibration with oligomycin, a phosphate burst occurs that is about half that seen after equilibration in the presence of high K⁺. Thus, the phosphate release data agree with the enzyme phosphorylation data in revealing an "intermediate" reactivity of the enzyme after equilibration with oligomycin in the absence of Na⁺ and K⁺.

The slow rate of phosphoenzyme accumulation following equilibration of enzyme with K⁺ and oligomycin may reflect the rate of oligomycin binding to the enzyme under turnover conditions. If this interpretation is correct, then the rate of formation of the inhibition complex can be estimated as equal to the rate of phosphoenzyme accumulation following equilibration with K⁺. We designed an experiment to compare the rate of accumulation of K⁺-insensitive E-P as a function of time of incubation with Na⁺ plus oligomycin (Fig. 4). Enzyme was first suspended in 100 mM Na⁺ to stabilize E₁. A two-stage rapid mixing protocol was followed. Enzyme plus Na⁺ was mixed with oligomycin for varying times before the second stage mixing with a solution containing K⁺ and [γ⁻³²P]ATP. The second stage reaction mixture was acid-quenched after a constant interval (37 ms). The rate of accumulation of K⁺-insensitive E-P was 5 s⁻¹ (open circles, Fig. 4), which is similar to the rate of phosphoenzyme accumulation subsequent to equilibration of enzyme with oligomycin in the presence of K⁺ (lower curve of Fig. 1). The presence of 20 mM KCl in the enzyme suspension from the beginning (closed circles, Fig. 4) reduces the total amount of E-P formed, presumably by increasing the ratio of E₂ to E₁, but has no effect on the rate of E-P accumulation. If Na⁺ is omitted until the second stage (triangles, Fig. 4), accumulation of E-P was undetectable over the 800 ms period of this stage. Over longer time intervals (minutes), oligomycin is evidently able to form an inhibited complex with the enzyme (closed circles, Fig. 1).

The rate of formation of the initial complex between enzyme and oligomycin should be linearly dependent on oligomycin concentration at sufficiently low concentrations of oligomycin. To test whether the rate of initial complex formation is rate-limiting for inhibition under the conditions of Fig. 3, the experiment was repeated using only 5 µg/ml of oligomycin.
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oligomycin (data not shown). The rate of phosphoenzyme accumulation was only 10% slower than the rate at the higher concentration of inhibitor (25 μg/ml). Thus, over this range of inhibitor concentrations, formation of the inhibition complex is evidently a slower step subsequent to oligomycin binding.

Dependence of the Inhibition on the Concentrations of Oligomycin and NaCl—The results presented so far make it clear that Na⁺ facilitates the development of oligomycin inhibition. In order to measure these concentration dependencies in more detail the enzyme was equilibrated with different concentrations of oligomycin either in the presence or the absence of Na⁺ and later phosphorylated by ATP in the presence of K⁺. When Na⁺ was absent from the preincubation medium, phosphorylation was carried out for 25 ms in order to optimize detection of the inhibition complex formed prior to phosphorylation (Fig. 1). Fig. 5 shows increase in the level of K⁺-insensitive E-P as a function of oligomycin concentration. The data fit a rectangular hyperbola with half-maximal increase in phosphoenzyme occurring at 8-9 μM/ml (open circles) when the initial equilibration of enzyme with oligomycin was carried out in the absence of Na⁺. This demonstrates that the concentrations of oligomycin in the experiments of Figs. 1–4 are saturating. When the enzyme was equilibrated with oli-

FIG. 5. Dependence of phosphoenzyme level on oligomycin concentration. Phosphorylation was used to monitor formation of the inhibition complex in the presence () or absence (○) of NaCl. O, the enzyme syringe contained oligomycin at the concentrations shown in the figure, 3 mM MgCl₂, and microsomes (1 mg/ml) with no added NaCl or KCl. The substrate syringe contained additional ligands to give final concentrations of 100 mM NaCl, 20 mM KCl, 3 mM MgCl₂, and 10 μM [γ-³⁵P]ATP. The enzyme was equilibrated with oligomycin for 5 min prior to the addition of substrate and phosphorylated for 25 ms. ○, the enzyme syringe contained oligomycin at the concentrations shown, microsomes (0.91 mg/ml), 3 mM MgCl₂, and 10 mM NaCl. The substrate syringe contained additional ligands to give final concentrations of 10 mM NaCl and 1 mM KCl. Equilibration with oligomycin was carried out for 1 min and phosphorylation for 100 ms. Oligomycin concentration was adjusted for each point by addition of aliquots in 95% ethanol to both the enzyme and substrate solutions.

TABLE 1
Characteristics of oligomycin and Na⁺ binding sites in Electrophorus (Na,K)-ATPase

The data of Figs. 5 and 6 were fitted to the Hill equation using MLAB (11). For oligomycin the concentrations producing half-maximal inhibition are expressed in micrograms per ml because the drug used was a mixture of the A, B, and C forms (Sigma).

<table>
<thead>
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<th>Ligand</th>
<th>Incubation condition</th>
<th>Kₗ₅</th>
<th>n</th>
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<td>Na⁺</td>
<td>−Oligomycin</td>
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<tr>
<td>Na⁺</td>
<td>+Oligomycin</td>
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<tr>
<td>Oligomycin</td>
<td>+Na⁺</td>
<td>1.85 μg/ml</td>
<td>1.06</td>
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FIG. 6. Dependence of the phosphoenzyme level on [NaCl] in the presence and absence of oligomycin. □, enzyme solution containing 3 mM MgCl₂, microsomes (1.03 mg/ml), oligomycin (30 μg/ml), and the indicated concentrations of NaCl was mixed with substrate solution with ligands added to give final concentrations of 100 mM NaCl, 20 mM KCl, 3 mM MgCl₂, and 10 μM [γ-³⁵P]ATP. Phosphorylation was allowed to proceed for 25 ms. ●, control experiment identical to the previous experiment except that the final concentration of NaCl was the same as the initial level, KCl and oligomycin were omitted, and the protein concentration was 1 mg/ml.

FIG. 7. Effect of oligomycin on the Na⁺-activated conversion of E₁ to E₂. Conversion of E₁ to E₂ was measured by incubating electrophores (Na,K)-ATPase with K⁺ for the indicated times prior to adding NaCl and ATP to initiate phosphorylation. ●, microsomes (1 mg/ml) suspended in the standard buffer with oligomycin (133 pg/ml), and no additional monovalent cations were mixed with 20 mM KCl, and incubated for the indicated times prior to mixing with 10 μM [γ-³⁵P]ATP and 100 mM NaCl. ●, microsomes (1.1 mg/ml) suspended in the standard buffer with 10 mM NaCl and oligomycin (50 μg/ml) were mixed with 20 mM KCl and subsequently with 10 μM [γ-³⁵P] ATP and 100 mM NaCl. Phosphorylation was carried out for 25 ms in both cases. Concentrations stated are those present after mixing. Where ligands were added early in the reaction their concentrations were held constant or increased as stated in the later stages.

gomycin in the presence of 10 mM Na⁺, the half-maximal increase in E-P occurred at 1.85 μg/ml (closed circles) and the concentration dependence was hyperbolic (Table I).

A similar type of experiment demonstrates the Na⁺ concentration dependence of the inhibition (Fig. 6). In the experiment represented by the open triangles, 50 μg/ml of oligomycin was equilibrated with enzyme in the presence of NaCl concentrations varying from 0 to 20 mM. Next, ATP was added together with K⁺ and sufficient Na⁺ to bring the final concentration to 100 mM. These data are characterized by a Hill coefficient of about 2.6. The closed circles demonstrate the Na⁺ dependence of the phosphorylation of the enzyme in the absence of oligomycin and K⁺. Note that the two sets of data have similar parameters (Table I).

Effect of Oligomycin on the Kinetics of the Transition of E₁ to E₂—We have previously shown (12) that the phosphorylation reaction can be used to monitor the kinetics of conformational transitions involving the Na⁺- and K⁺-stabilized
states of the nonphosphorylated enzyme. This technique was used in the present study to determine if the rate of conversion of $E_1$ to $E_2$ is affected by oligomycin. In one experiment (Fig. 7, closed circles), enzyme was equilibrated with oligomycin and $3 \text{ mM} \text{ MgCl}_2$ and mixed in the first stage with $50 \text{ mM} \text{ KCl}$ to initiate conversion of $E_1$ to $E_2$. After varied times in the first stage, the second stage was initiated by mixing with $10 \text{ \mu M} \text{ ATP}$ and $100 \text{ mM} \text{ NaCl}$. Phosphorylation proceeded for $25 \text{ ms}$ before acid quenching. The amount of phosphoenzyme decreased monophasically as a function of time of exposure to $\text{KCl}$. The rate constant for this decay was $30 \text{ s}^{-1}$, approximately half the rate of decay in the absence of oligomycin. The apparent decline in the rate of conversion of $E_1$ to $E_2$ suggests that dissociation of oligomycin might be rate-limiting.

When (Na,K)-ATPase is equilibrated with oligomycin, $3 \text{ mM} \text{ MgCl}_2$ and $100 \text{ mM} \text{ NaCl}$ before mixing with $K^+$ (Fig. 7, triangles), a much slower conversion of $E_1$ to $E_2$ occurs ($0.3 \text{ s}^{-1}$). Although not shown in the figure, the $K^+$-insensitive $E-P$ in this experiment decayed to $0.125 \text{ nmol/mg}$ after $10 \text{ s}$. The results of Fig. 7 support our earlier conclusion that there is an interaction of oligomycin with enzyme and $\text{Mg}^{2+}$ in the absence of $Na^+$ and further demonstrate that $Na^+$ enhances the apparent affinity of the enzyme for oligomycin by decreasing the dissociation rate.

**DISCUSSION**

These experiments demonstrate that sodium ions, in acting on the (Na,K)-ATPase to enhance oligomycin binding and to activate enzyme phosphorylation, display similar apparent affinities. $Na^+$ acts to increase the rate of formation of the inhibited complex of enzyme with oligomycin (Fig. 4) and to slow its rate of dissociation (Fig. 7). $K^+$ effects are opposite to those of $Na^+$ in these respects. In the absence of both $Na^+$ and $K^+$, oligomycin appears to form a readily dissociable complex with the enzyme (Fig. 7), but the effects of this interaction are restricted to half or less of the total enzyme phosphorylation sites (Fig. 1). Titration of the enzyme with oligomycin did not reveal binding site heterogeneity (Fig. 5).

A model which does not require homologous site interactions can be proposed: binding of oligomycin to a single site might form an initial complex which retains full catalytic activity; a subsequent transition would then be required to inhibit the enzyme. To account for the observed heterogeneity in the absence of $Na^+$ and $K^+$ and in the presence of saturating oligomycin (Fig. 1), these two conformers must be in equilibrium and of approximately equal stability:

$$E_1 + \text{oligomycin} \rightleftharpoons E_1(\text{oligo}) \rightleftharpoons E_1'(\text{oligo})$$

where $K_2 = 1$.

(1) (active)

(2) (inactive)

Under these conditions, simultaneous addition of substrate, $Na^+$, and $K^+$ would initiate a rapid phase of $K^+$-insensitive phosphorylation of $E_1'(\text{oligo})$ and an approximately equal burst of phosphate release resulting from the turnover of $E_1'(\text{oligo})$. With successive turnover cycles, more $E_1'(\text{oligo})$ could form, transform to $E_1'(\text{oligo})$, and produce more $K^+$-insensitive $E-P$ in the slow phase. This model is consistent with the results of Figs. 1, 3, and 7.

To encompass the observed effects of monovalent cations on this system, a different pathway must become significant in the presence of $Na^+$:

$$Na^+ \cdot E_1 + \text{oligomycin} \rightleftharpoons Na^+ \cdot E_1(\text{oligo}) \rightleftharpoons Na^+ \cdot E_1'(\text{oligo}).$$

Fig. 7 demonstrates that the inhibitory complex formed in the presence of $Na^+$ is much more stable than that formed in its absence. In addition, $Na^+$ increased the rate of formation and level of the $K^+$-insensitive phosphoenzyme while inhibiting the formation of the $P$-burst (Fig. 3), suggesting that it stabilizes the conformer that binds oligomycin. If the observed $Na^+$-dependent rates of formation ($4-5 \text{ s}^{-1}$) and dissociation ($0.3 \text{ s}^{-1}$) of the inhibition complex correspond to the forward and reverse reactions in step 4, then the equilibrium will be shifted strongly in the direction of the inactive complex, in agreement with the observed behavior. The antagonistic effect of $K^+$ toward oligomycin inhibition is adequately explained by its accepted role in stabilizing $E_2$.

One aspect of these experiments seems inconsistent with the postulated equilibrium between $E_1'(\text{oligo})$ and $E_1''(\text{oligo})$. It is apparent that if $K_2 = 1$, there will be an equal distribution of active and inactive states, in agreement with the results obtained in Fig. 1 in the absence of $Na^+$ and $K^+$. However, the reverse rate constant for step 2 would be expected to agree with that measured in Fig. 7 (closed circles), i.e. $30 \text{ s}^{-1}$, whereas the forward rate constant for step 2 is of the order of minutes, resulting in an equilibrium strongly favoring the active state. Several modifications of this model might explain the discrepancy between the apparent and predicted amounts of active state. One possibility is that the formation of the inactive complex in the $Na^+$-independent pathway is rate-limited by a step which precedes the binding reaction. This might be a slow structural transformation that shifts the oligomycin binding site from an occluded to an open conformation that is stabilized by oligomycin. In this case, $k_2$ might actually be comparable to $k_{-s}$, so that after a delay of minutes the active and inactive states become equally populated. Sodium ion would therefore elicit two effects: 1) an increase in the rate of the conformation change exposing the inhibitor site, and 2) an increase in the affinity of the enzyme for oligomycin. The first effect might eliminate the dependence of the rate of oligomycin binding on the slow structural transformation in the $Na^+$-independent pathway thus allowing the true rate of formation of the inactive complex to be measured ($4-5 \text{ s}^{-1}$). A second possibility is that dissociation of oligomycin in the $Na^+$-independent pathway is extremely slow (order of minutes) and that $K^+$ greatly accelerates this dissociation. That $K^+$ does not appear to accelerate the breakdown of $Na^+ \cdot E_1''(\text{oligo})$ does not necessarily contradict this assumption since $Na^+$ may antagonize the action of $K^+$. The possibility that $Na^+$ and $K^+$ influence the kinetics of the reactions involved in the formation and breakdown of the inactive inhibitor complex makes it difficult to distinguish between these alternative mechanisms.

In view of this difficulty with a model involving a single catalytic site, some consideration must be given to a dimeric or interacting site model as indicated above. If oligomycin binds tightly to the conformational state stabilized by $Na^+$ and weakly or not at all to the $K^+$-stabilized conformation as our results indicate, then the observed pattern of inhibition in the presence of $Mg^{2+}$ suggests a dimer in which the conformation of one-half of the molecule is $E_1$, while that of the other half is $E_2$. Addition of $Na^+$ to this hybrid state presumably drives both subunits into the $Na^+$-stabilized conformation resulting in a tightly bound oligomycin/catalytic subunit and complete inhibition of enzyme activity:

$$E_1 \cdot K/E_1 \cdot K \rightleftharpoons E_1(\text{oligo}) \rightleftharpoons Na^+ \cdot E_1(\text{oligo}) \rightleftharpoons Na^+ \cdot E_1(\text{oligo})$$

Similarly, $K^+$ binding in the absence of $Na^+$ might shift the conformational equilibrium in the direction of an $E_2$ dimer preventing oligomycin from binding to the enzyme. The inhibitor binding site conformation must be different in $E_1(\text{oligo})$ and $Na^+ \cdot E_1(\text{oligo})$ in order to account for the fact that these states are driven back to the $E_2$ conformation by...
K+ at different rates (Fig. 7). A similar conclusion applies to $E_1$ and $E_1$-Na which react with oligomycin at distinctly different rates (Fig. 4).

It is unclear from the present results whether the apparent half-of-the-site reactivity towards oligomycin observed in the absence of Na+ and K+ results from asymmetric dimerization of identical subunits or is induced by the binding of oligomycin to only half of the subunits (16). If the subunits are in different conformational states prior to exposure to oligomycin, conversion of the $E_2$ half of the dimer to $E_2$ must be faster than phosphorylation to account for the fact that $E$-P formation following preincubation with Mg2+ obeys simple first order kinetics (Fig. 2A). This does not conflict with previous results indicating the presence of a slow rate of conversion of $E_2$ to $E_2$ (7, 12, 17) since those measurements were carried out following an initial exposure to K+ which forms a stable occluded complex with the enzyme (18, 19).

A further question that arises in connection with the dimer hypothesis concerns the extent of chemical or conformational coupling between subunits during ATP hydrolysis. In the half-of-the-site mechanism proposed by Stein et al. (20) the catalytic subunits of an $\alpha_2\beta_2$ tetramer are assumed to cycle 180° out of phase with the consequence that phosphorylation of one $\alpha$ chain is coupled to dephosphorylation of the adjacent $\alpha$ chain. Two observations suggest that tight coupling of catalytic activity between the subunits does not occur in the partially inhibited enzyme following the addition of ATP: First, addition of ATP, Na+, and K+ to the oligomycin-treated enzyme produced a rapid phase of P7 liberation indicating that the uninhibited enzyme is able to complete at least one rapid cycle of ATP hydrolysis before being trapped in $E$-P. If the subunits had been coupled in the manner proposed by Stein et al., then inhibition of the phosphoenzyme conformational transition in one subunit should have blocked the $E_2 \rightarrow E_1$ conversion in the adjacent subunit preventing rapid ATP hydrolysis by the latter. Second, the slow increase in $E$-P level which follows the rapid phase of phosphorylation in Fig. 1 leads to the formation of a doubly phosphorylated state which is excluded by the model. The fact that this behavior occurs in the presence of oligomycin does not exclude the existence of a different type of coupling under physiological conditions. For example, our previous studies (21) have shown that as the ATP concentration is raised in the presence of saturating Na+ and K+, $E$-P formation approaches half of the maximal level, consistent with the model of Stein et al.

The results of Fig. 7 are in agreement with those of Karlisch et al. (7), indicating that oligomycin slows the rate of conversion of $E_2$-Na to $E_2$-K but does not affect the rate of the $E_2$-K to $E_1$-Na reaction and with the conclusion of Sachs (15) that oligomycin does not combine with any $E_2$ form of the enzyme. Robinson and Mercer (22) found that oligomycin decreased vanadate binding (to 56% of control value) in the presence of MgCl2 and suggested that divalent cations select for the $E_2$ state whereas oligomycin selects for $E_1$. Our previous results (12) have indicated that enzyme preincubated with MgCl2 behaves much like $E_1$ in the transient state, whereas the tryptic digestion studies of Castro and Farley (23) suggest that the Mg2+-treated enzyme is similar to the K+ stabilized form (but see Ref. 24). Other investigators have noted "inhibitory" effects of MgCl2 indicating stabilization of $E_2$. Forgac (25), for example, has observed an Mg2+-stabilized state in a slightly more purified Electrophorus enzyme which had a $K_d$ for Mg2+ of about 1 mM in the absence of other ligands, and which seemed to be distinct from $E_2$-K or $E_1$-Na. The state described in the present work does not depend upon the presence of Mg2+ in the medium, and may thus be different in some respects from those described by other investigators. It is clear that the enzyme behaves differently in the absence of Na+ and K+ than either the $E_1$-Na or $E_2$-K states. To reconcile these differences, we suggest a model in which some state intermediate to $E_1$ and $E_2$ is present which can be rapidly converted to $E_1$ and phosphorylated when Na+ and ATP are added (unlike $E_2$-K), but which does not bind oligomycin very well (unlike $E_1$-Na).

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