The Kinetics of Adenylosuccinate Lyase*

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SUMMARY

Initial rate kinetics, product inhibition patterns, and equilibrium kinetics all indicate that cleavage of adenylosuccinate (AMP-S) by yeast adenylosuccinate lyase proceeds primarily by a reaction in which fumarate leaves the enzyme before AMP and cannot bind to free enzyme at kinetically significant concentrations. The alternate path, in which AMP leaves first, could be detected at equilibrium by the existence of AMP$^\rightarrow$AMP-S exchange at saturating levels of fumarate and AMP-S. This exchange was less than one-tenth the rate of fumarate$^\rightarrow$AMP-S exchange. Moreover, evidence was obtained that the high concentrations of fumarate and AMP-S enhanced the AMP exchange rate in a manner unrelated to their roles as substrates. Thus, under steady state conditions, the contribution of the alternate path must be very small.

The steady state analysis also provided evidence that breakages of the C$-\text{N}$ and C$-\text{H}$ bonds of the substrate are not the slowest steps of the over-all reaction.

The inability of free enzyme to bind fumarate at kinetically significant concentrations is supported by failure of other dicarboxylic acids to inhibit the enzyme. However, the succinyl group of AMP-S plays a role in attachment to the enzyme, since AMP-S binds 8 times more strongly than AMP, as indicated by their $K_\text{m}$ values. It is suggested that the free enzyme exists principally in a conformation that does not bind dicarboxylic acids, but that attachment of AMP or of the AMP portion of AMP-S promotes conversion to a form that can bind fumarate or the succinyl portion of AMP-S. The resultant inability of the enzyme to be inhibited by dicarboxylic acids may be of biological value.

In earlier publications (1, 2) we have reported that adenylosuccinate lyase ((EC 4.3.2.2), AMP$^\rightarrow$AMP$^\rightarrow$fumarate) is inhibited by $p$-chloromercuriphenyl sulfonate and that the enzyme is protected completely from this reagent by the presence of the substrates adenylosuccinate and AMP, but not by fumarate at concentrations up to 12 times the $K_\text{m}$ value. This suggested that the free enzyme may be unable to bind fumarate to a significant extent, i.e. there may be an obligatory order of attachment of AMP and fumarate. The present report describes a study of the steady state kinetics of the reaction in both directions, including product inhibition, and a study of isotope exchange at equilibrium. In addition, the effects of several analogues of the substrates have been examined. These approaches yield information about the sequence of release of AMP and fumarate from the enzyme.

The diagnostic value of initial rate kinetics and product inhibition studies in elucidating the sequence of combination of substrates and products with enzymes has been pointed out by Alberty (3) and, more explicitly, by Cleland (4). In the case of a simple uni-bi reaction, such as that catalyzed by adenylosuccinate lyase, an ordered sequence of release of product molecules should be characterized by product inhibition which is competitive in the case of the last product to leave, but not competitive in the case of the first product to leave. However, with the development of additional criteria for ordered reactions, such as the isotope exchange kinetics at equilibrium (5), examples have been reported of enzymes in which the kinetics of the net reaction indicates an ordered sequence, while the kinetics at equilibrium reveals alternate paths (6, 7). Several possible explanations for such discrepancies have been suggested (8, 9). The simplest explanation, as recently pointed out by Ray and Roselli (10), is that the kinetics of the net reaction cannot be studied with sufficient precision to detect the participation of a minor path in the enzyme mechanism. For this reason it is desirable to use both methods of analysis.

EXPERIMENTAL PROCEDURE

Reagents—Adenylosuccinate was prepared enzymically from AMP and fumarate (11). Fumaric acid (Fluka) and ammonium sulfate were recrystallized from dilute EDTA and then

1 The abbreviations used are: AMP-S, adenylosuccinate; E-AMP and E-AMP-S, binary complexes of enzyme with AMP and AMP-S, respectively; E-AMP-F, ternary complex of enzyme, AMP, and fumarate.
from hot water. $^{14}C$-Fumarate was obtained from Merck, Sharp and Dohme Company, Montreal, Quebec, and was found to contain a trace of radioactive impurity with chromatographic behavior similar to that of AMP-S. The contamination was small enough so that simple control experiments could be used to correct for it (see "$^{14}C$-Fumarate $\Rightarrow$ AMP-S Exchange," below). $^{14}C$-AMP was obtained from Schwarz Biores. Purine nucleotide was a gift from Dr. A. Hampton.

**Yeast Adenylosuccinate**—During attempts to purify adenylosuccinate lyase it became apparent that the enzyme is sensitive to trace heavy metals (1) and that maximal recovery and stability could be obtained if all procedures were carried out in the presence of EDTA. The procedure adopted differs in certain respects from those previously reported.

Dried bakers' yeast (1 kg) was allowed to autolyze for 7 hours at room temperature in 3 liters of 0.1 M NaHCO₃. After centrifugation (20 min at 5,000 $\times g$), the supernatant was made 1 M with respect to EDTA, 38 g of ammonium sulfate were added per 100 ml, and the mixture was stirred for 30 min. The precipitate was collected by centrifugation (20 min at 5,000 $\times g$) and dissolved in a minimal amount of 2 mM EDTA, pH 8 (about 300 ml), and 13 g of ammonium sulfate were added per 100 ml of the enzyme solution. The mixture was stirred for 30 min at room temperature and clarified by centrifugation (20 min at 17,000 $\times g$), the supernatant was made 1 M with respect to EDTA, 38 g of ammonium sulfate were added per 100 ml of supernatant. The mixture was again stirred for 30 min at room temperature and centrifuged at 17,000 $\times g$ for 20 min. The residue, which contained the enzyme, was dissolved in 120 ml of 5 mM EDTA (pH 8). The enzyme solution was brought to 60$^\circ$ in a water bath, kept at this temperature for 31 min, chilled, and centrifuged (20 min at 17,000 $\times g$). The supernatant contained about 60% of the original activity at a specific activity 10 times that of the original autolysate. The enzyme solution from the heat treatment was dialyzed against three changes of 5 mM potassium phosphate and 2 mM EDTA at pH 6.8, and was applied to a column (2 x 40 cm) of DEAE-cellulose which had been washed by filtration successively with EDTA, NaOH, and water and then adjusted to pH 6.8. Elution was accomplished with the use of a variable gradient device (12). The two vessels closest to the column each contained 350 ml of 0.01 M potassium-EDTA at pH 6.8, and the last two vessels each contained 350 ml of 0.05 M potassium-EDTA at pH 6.0. The enzyme emerged from the column after approximately 1 liter of eluting agent had passed through. The enzyme was precipitated by adding 40 g of ammonium sulfate per 100 ml of enzyme solution, centrifuged at 17,000 $\times g$ for 20 min, and dissolved in 2.5 ml of 1 mM EDTA (pH 7). It was desalted by passage through a column of Sephadex G-25 (bed volume, 50 ml) which had been equilibrated with 5 mM potassium phosphate and 2 mM EDTA at pH 6.8. The enzyme solution was then applied to a second DEAE-cellulose column (1.5 x 16 cm). The elution gradient was prepared from 200 ml of 0.01 M potassium phosphate (pH 6.8) in each of the four vessels nearest the column, and 200 ml of 0.05 M sodium-EDTA (pH 6.0) in the fifth vessel. The enzyme emerged after about 850 ml of effluent had run off the column. The tubes containing enzyme were pooled, and the specific activity was found to be 200 to 400 times that of the original autolyzate. The solution was adjusted to pH 8.0 with 1 M Tris and was frozen. This preparation was found to be very stable at room temperature.

**Rate Measurement**—The rate of appearance or disappearance of adenylosuccinate was determined by measuring the rate of increase or decrease of absorbance at 280 m$\mu$ (11) with a Cary model 15 recording spectrophotometer equipped with both 0 to 1 and 0 to 0.1 absorbance slide-wires. For rate measurements at low substrate concentrations, a cuvette with a 5-cm light path was used. Additional sensitivity was obtained by using the expanded slide-wire of the spectrophotometer so that accurate rate determinations could be made within the first 5% of the reaction. The reaction was carried out at 25$^\circ \pm 0.1$.

The reaction media contained 20 mM Tris-chloride and 20 mM EDTA at pH 7.8 except where otherwise stated.

All reciprocal plots and replots were constructed by computer (13) with programs kindly furnished by Dr. W. W. Cleland.

$^{14}C$-AMP $\Rightarrow$ AMP-S Exchange—The effect of concentration of fumarate and AMP-S (in a fixed ratio) on the rate of interconversion of AMP and AMP-S at equilibrium was determined as follows. Various concentrations of fumarate and AMP-S (in the ratio, 70:1) were combined with 0.1 mM unlabeled AMP, 12 mM Tris-chloride (pH 7.8), 24 mM KCl, 0.3 mM EDTA, and an amount of enzyme consistent with linear exchange kinetics for at least 3 min. The total volume was 500 ml. The samples were incubated at 25$^\circ$ for 24 hours to ensure equilibrium, after which 10 ml of 0.2 mM 8-14C-AMP (21 mC per mmole) were added. The reaction was stopped after 3 min of incubation at 25$^\circ$ by the addition of 100 $\mu$moles of HCl. The AMP-S in the sample was then isolated by cation exchange chromatography as described below and its radioactivity was measured.

$^{14}C$-Fumarate $\Rightarrow$ AMP-S Exchange—The effect of concentration of fumarate and AMP-S (in a fixed ratio) upon the rate of interconversion of fumarate and AMP-S at equilibrium was determined as follows. Various concentrations of AMP-S and AMP (in the ratio, 2:1) were combined with 14 mM fumarate, 12 mM Tris-chloride (pH 7.8), 24 mM KCl, 0.3 mM EDTA, and an amount of enzyme consistent with linear exchange kinetics for at least 40 min. After complete equilibration at 25$^\circ$, 4 $\mu$l of 82 mM 14C-fumarate (specific activity, 60 mC per mmole) were added to each tube. The tubes were then incubated for a further 40 min at 25$^\circ$, and the reaction was stopped by the addition of 100 $\mu$l of 0.4 N HCl. The AMP-S was then isolated and counted. A control tube in which the enzyme was omitted was regularly included in the experiments because the 14C-fumarate contained a trace contaminant with chromatographic behavior similar to that of AMP-S. The radioactivity contributed by this impurity was reproducibly less than 10% of the total radioactivity of the isolated AMP-S, and was subtracted from the total radioactivities.

Preliminary experiments showed that under the experimental conditions used the rates of exchange of both 14C-fumarate and 14C-AMP into AMP-S were constant with respect to time, indicating that neither net reaction nor approach to isotopic equilibration was sufficient to influence the observed rates of exchange.

**Isolation of AMP-S**—The separation of AMP-S, AMP, and fumarate in the incubation mixtures of the isotopic exchange experiments was performed by a modification of the method of Katz and Comb (14) for the separation of nucleotides by cation exchange chromatography. A sample (500 $\mu$l) of each $\theta$.

# Rectangular cuvettes with internal dimensions 5 cm long, 0.5 cm wide, and 4.3 cm high were obtained from Pyrocell Manufacturing Company, Westwood, New Jersey.
FIG. 1 (left). Inhibition of AMP-S cleavage by AMP. The inset shows the replotted slope with respect to [AMP]. Enzyme assays were carried out as described under "Experimental Procedure." Rates are expressed as change in the micromolar concentration per 23 min.

FIG. 2 (right). Inhibition of AMP-S cleavage by fumarate (FU). The inset shows replots of slope and intercept (INT.) with respect to [fumarate]. Enzyme assays were carried out as described under "Experimental Procedure." Rates are expressed as change in the micromolar concentration per 21 min.

A 2-ml sample of the AMP-S solution was added to 15 ml of phosphor solution (7 g of 2,5-diphenyloxazole (PPO), 0.3 g of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene (dimethyl-POP), and 100 g of naphthalene per liter of dioxane) and counted with a scintillation counter.

RESULTS

Product Inhibition—The effect of AMP on the rate of cleavage of adenylosuccinate is shown in Fig. 1. The inhibition is clearly competitive over a wide range of AMP concentrations (to 60 times the $K_i$ value), and when the slopes of the reciprocal plots are replotted against the AMP concentration a straight line is obtained over a 50-fold range of slope, as shown in the inset. The competitive inhibition and the linear replot both indicate that AlIP inhibits by combining only with free enzyme. In contrast, the presence of fumarate causes a change in the intercept as well as in the slope of the reciprocal plot (Fig. 2), showing that fumarate combines with an enzyme form other than free enzyme. Both the slope and intercept are linear functions of the fumarate concentration, as shown in the inset. This indicates that fumarate, at the concentrations used (up to 3 $K_f$) does not combine with free enzyme to an appreciable extent. The product inhibition data are consistent with the ordered mechanism shown in Scheme 1.

Initial Rates of Reverse Reaction—The kinetic data for the reverse direction are shown in Fig. 3. The usable ranges of concentration of substrates were somewhat limited by the position of the equilibrium. Thus, at concentrations near the $K_m$ values, equilibrium was approached before sufficient reaction occurred to be measured accurately, despite the high sensitivity of the assay. For this reason, only approximate values for the Michaelis constants for fumarate and AMP at this pH can be determined. Nevertheless, the reciprocal plots are reasonably linear and intersect at a value of AMP close to that corresponding to the $K_{AMP}$ determined from the product inhibition studies, as predicted if AMP is the first substrate bound (10).

The kinetic constants obtained from the data of Figs. 1 to 3 are listed in Table I. As defined by the rate equation, $K_F$ and $K_{AMP}$ are the $K_m$ values at infinite concentration of the other substrate. When these values are used to calculate the equilibrium constant, according to the two Haldane relationships for an ordered mechanism, values of 7.3 mM and 9.8 mM are obtained, in agreement with the value of 0.8 mM obtained by direct measurement of the equilibrium constant (11). This agreement lends support to the ordered mechanism and indicates that the inhibitions obtained with AMP and fumarate are due principally to their roles as products, i.e. to the combination of each with a single form of the enzyme and at a single site.

From these data, assuming that Scheme 1 represents the principal reaction path, it is possible to compare the rates of attachment and release of AMP-S and AMP. For example, $k_F/k_1 = K_{AMP}/K_F = 4.6$, and $k_F/k_2 = K_F/K_{AMP} = 0.58$. Thus AMP leaves the enzyme 4 to 5 times faster than...
TABLE I

<table>
<thead>
<tr>
<th>Kinetic constants of adenylosuccinate lyase at pH 7.8</th>
</tr>
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<tbody>
<tr>
<td>The kinetic constants, which were measured under conditions</td>
</tr>
<tr>
<td>where ( V_1/V_2 = 6.5 ), are those defined by Cleland (4) for ordered</td>
</tr>
<tr>
<td>mechanisms (Scheme 1) for which the rate equation is</td>
</tr>
</tbody>
</table>
| \[
| v = \frac{V_1V_2([AMP-S] - [F][AMP]/K_{eq})}{V_3K_{AMP-S} + V_3[AMP-S] + \frac{K_{AMP}V_1[F]}{K_{eq}} + \frac{K_FV_1[AMP]}{K_{eq}} + \frac{V_1[F][AMP]}{K_{eq}} + \frac{V_1d[AMP-S][F]}{K_{IF}}}
| \]
<p>| |
| |</p>
<table>
<thead>
<tr>
<th>Defining equation</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>( K_{AMP-S} )</td>
<td>( \frac{k_0(k_1 + k_{-1})}{k_0(k_2 + k_3)} )</td>
</tr>
<tr>
<td>( K_{AMP} )</td>
<td>( \frac{k_{-1}}{k_3} )</td>
</tr>
<tr>
<td>( K_F )</td>
<td>( \frac{k_2 + k_{-2}}{k_2} )</td>
</tr>
<tr>
<td>( K_{AMP-S} )</td>
<td>( \frac{k_1}{k_1} )</td>
</tr>
<tr>
<td>( K_{AMP} )</td>
<td>( \frac{k_2}{k_3} )</td>
</tr>
<tr>
<td>( K_{IF} )</td>
<td>( \frac{k_3 + k_1}{k_2} )</td>
</tr>
<tr>
<td>( K_{eq} )</td>
<td>( \frac{V_1K_{AMP}}{V_3K_{AMP-S}} )</td>
</tr>
<tr>
<td>( K_{eq} )</td>
<td>( \frac{V_1K_{AMP}}{V_3K_{AMP-S}} )</td>
</tr>
<tr>
<td>( K_{eq} )</td>
<td>( \frac{[AMP][fumarate]}{[AMP-S]} )</td>
</tr>
</tbody>
</table>

\( K_{AMP} \) does AMP-S, whereas there is only a small difference between the two nucleotides in rate of attachment. In addition, it can be shown that the rate constants of fumarate dissociation and AMP dissociation are approximately the same \( (k_0 - k_3) \). Since \( k_3 \) is a complex rate constant which includes the rate constants for the breakage of two covalent bonds, a C—N bond and a C—H bond, the rate constants for the bond-breaking step must be at least as large as \( k_3 \) and therefore at least as large as \( k_0 \). It can be concluded, therefore, that it is not the breakage of either of these bonds that is the slowest step in the over-all reaction.

In the sequence, \( E \cdot AMP-S \overset{k_0}{\underset{k_{-3}}{\longrightarrow}} E \cdot AMP \overset{k'}{\longrightarrow} E \cdot AMP + fumarate \), the over-all rate constant, \( k_2 \), is related to the component rate constants as follows

\[
k_2 = \frac{k_0k'}{k_0 + k_{-3} + k'}
\]

Thus, \( k_2 \) cannot exceed the lowest rate constant of the individual forward reactions.

TABLE II

<table>
<thead>
<tr>
<th>Effects of purine nucleotide analogues on cleavage of AMP-S</th>
</tr>
</thead>
<tbody>
<tr>
<td>The cuvettes contained, in a final volume of 0.4 ml, 0.021 m</td>
</tr>
<tr>
<td>potassium phosphate, pH 7.6, 0.08 mM AMP-S, the compounds to</td>
</tr>
<tr>
<td>be tested, and enzyme.</td>
</tr>
<tr>
<td>Analogue</td>
</tr>
<tr>
<td>----------</td>
</tr>
<tr>
<td>AMP</td>
</tr>
<tr>
<td>Purine nucleotide</td>
</tr>
<tr>
<td>IMP</td>
</tr>
<tr>
<td>Thio-IMP</td>
</tr>
<tr>
<td>Adenosine</td>
</tr>
<tr>
<td>ATP</td>
</tr>
</tbody>
</table>

Effects of Analogues of Substrates—The effects of structural analogues of AMP and fumarate on the rate of the reaction in the forward direction cast some light on the interaction of the substrates with the enzyme. The effects of various analogues of AMP are shown in Table II. The small degree of inhibition by either ATP or adenosine indicates that the monophosphate group is important in the binding of substrate to the enzyme. The 6 amino group is also important, as seen from the poor inhibition by purine nucleotide, by IMP, and by thio-IMP (6-mercaptopurine nucleotide). It appears, therefore, that the enzyme is specific for the 6-amino group or for the electronic configuration conferred upon the purine ring by such a group.

Of the dicarboxylic acids tested as inhibitors, mesaconate (2-methylfumarate) most closely resembles fumarate in structure. The others do not have the planar structure of fumarate, but are similar to the dicarboxylic acid portion of adenylosuccinate. As shown in Table III, none of the dicarboxylic acid analogues tested inhibited the reaction in either the forward or the reverse direction, even at quite high concentrations.

Isotope Exchange at Equilibrium—The equilibrium rates of \( ^{14}C \cdot AMP \rightleftharpoons AMP-S \) exchange and \( ^{14}C \cdot fumarate \rightleftharpoons AMP-S \) exchange were measured in separate experiments at fixed, high concentrations of the radioactive components: 37 times the \( K_{AMP} \) value and 35 times the \( K_F \) value, respectively. The two nonisotopic components were varied in a fixed ratio such that equilibrium conditions prevailed. According to Scheme 1, the fumarate \( \rightleftharpoons AMP-S \) exchange reaction should approach a maximal value as AMP and AMP-S are increased toward infinity, since the enzyme would be converted by these substrates into those forms required to catalyze the exchange, namely \( E \cdot AMP \) and \( E \cdot AMP-S \). On the other hand, the AMP \( \rightleftharpoons AMP-S \) rate would be expected to increase to a maximum and then to decrease toward zero as fumarate and AMP-S are increased toward infinity, because these substrates would drive the enzyme to the ternary complex, which, according to Scheme 1, is unable to release AMP. In addition, the maximal AMP \( \rightleftharpoons AMP-S \) rate

\( Although thio-IMP has been reported to inhibit the enzyme (16, 17), it does not inhibit if care is taken to eliminate heavy metals from the assay medium. Heavy metal complexes of thio-IMP are very potent inhibitors of the enzyme (1, 18).

\( The high concentrations of these substrates permitted the use of AMP-S concentrations high enough for a reasonable extent of incorporation of label into AMP-S well before approach to isotopic equilibrium.
Lack of inhibition by dicarboxylic acid analogues

Two assay conditions were used, measuring the rate in both the forward and reverse directions. For Assay 1 the cuvette contained, in a final volume of 0.4 ml, 0.021 M potassium phosphate, pH 7.6, 0.08 mM adenylosuccinate, potassium salts of the compounds tested, and enzyme. For Assay 2 the cuvette contained, in a final volume of 0.43 ml, 0.02 M potassium phosphate, pH 6.4, 0.07 mM AMP, 0.2 mM fumarate, the potassium salt of the compound tested, and enzyme.

<table>
<thead>
<tr>
<th>Compound tested</th>
<th>Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 mM fumarate</td>
<td>35 1</td>
</tr>
<tr>
<td>2 mM malate</td>
<td>0 1</td>
</tr>
<tr>
<td>1.2 mM malate</td>
<td>2 0</td>
</tr>
<tr>
<td>2 mM aspartate</td>
<td>0 1</td>
</tr>
<tr>
<td>2 mM N-acetylaspartate</td>
<td>0 1</td>
</tr>
<tr>
<td>1.2 mM N-acetylaspartate</td>
<td>0 2</td>
</tr>
<tr>
<td>2 mM succinate</td>
<td>0 1</td>
</tr>
<tr>
<td>12 mM succinate</td>
<td>0 2</td>
</tr>
<tr>
<td>2 mM mesaconate</td>
<td>0 2</td>
</tr>
<tr>
<td>1.2 mM mesaconate</td>
<td>0 2</td>
</tr>
<tr>
<td>1.2 mM thiomalate</td>
<td>0 1</td>
</tr>
</tbody>
</table>

Table III

FIG. 4. A (upper), 14C-fumarate ⇄ AMP-S exchange rates at equilibrium as a function of AMP concentration. The experimental conditions are described under "Experimental Procedure." Rates are expressed relative to the maximal fumarate ⇄ AMP-S exchange rate predicted by Scheme 1 (see Footnote 6). B (lower), 14C-AMP ⇄ AMP-S exchange rate at equilibrium as a function of fumarate concentration. Rates are expressed relative to the maximal fumarate ⇄ AMP-S exchange rate predicted by Scheme 1.

\begin{align*}
R_{F_{\text{max}}} & = \frac{k_{-2}k_{2}}{k_{-1} + k_{2}} [E] \\
\end{align*}

Therefore

\begin{align*}
\frac{1}{R_{F_{\text{max}}}} & = \frac{1}{k_{-1} [E]} + \frac{1}{k_{2} [E]} \\
\end{align*}

Since \( k_{-1} [E] = V_{z} \) and \( k_{2} = k_{3} = 4.6 \),

\begin{align*}
\frac{1}{R_{F_{\text{max}}}} & = \frac{1}{V_{z}} + \frac{1}{4.6V_{z}} \\
\end{align*}

where \( V_{z} \) is \( V_{\text{max}} \) in the direction of synthesis. It is therefore possible to calculate \( R_{F_{\text{max}}} \) from the measured \( V_{z} \) for the quantity of enzyme used in measuring the exchange reaction.
Further evidence for Scheme 2 is indicated in a double reciprocal plot of the fumarate vs AMP-S data (Fig. 5). This plot is not a straight line. The increased effectiveness of AMP seen at the highest concentrations can be explained by E-fumarate being formed at the high fumarate concentration used. Then, at the lowest AMP levels, fumarate exchange would occur principally via the E-fumarate route, but would shift to the upper, faster path at AMP concentrations high enough to compete with fumarate for free enzyme.

**DISCUSSION**

The steady state kinetic data obtained in these studies are in excellent agreement with those expected for the ordered mechanism (Scheme 1). As has been shown by Cleland (4), the rate law for this scheme (see Table I) reduces to the following two equations when either AMP or fumarate is used as inhibitor.

**Vary AMP-S, inhibit with fumarate**

\[
\frac{1}{v} = \frac{K_{\text{AMP-S}}}{V_1} \left( 1 + \frac{[\text{AMP}][F]}{K_{\text{AMP-S}}[F]} \right) \frac{1}{[\text{AMP}-S]} + \frac{1}{V_1} \left( 1 + \frac{[F]}{K_{IF}} \right)
\]

**Vary AMP-S, inhibit with AMP**

\[
\frac{1}{v} = \frac{K_{\text{AMP-S}}}{V_1} \left( 1 + \frac{[\text{AMP}]}{K_{\text{AMP}}} \right) \frac{1}{[\text{AMP}-S]} + \frac{1}{V_1}
\]

According to the ordered mechanism, therefore, AMP will be a competitive inhibitor, fumarate will be a noncompetitive inhibitor, and the slopes and intercepts of the reciprocal plots will be linear functions of the inhibitor concentration. These predictions are fulfilled by the experimental results. Moreover, the close agreement between \( K_{\text{AMP}} \) measured by initial rate studies and product inhibition and the agreement between the two Haldane relationships for the ordered mechanism both support the conclusion that under steady state conditions the mechanism is virtually ordered.

The equilibrium exchange data, however, while confirming that Scheme 1 represents the major path, indicate that this path is not absolutely compulsory and that AMP can leave before fumarate to a small extent under certain circumstances. According to the strictly ordered mechanism, AMP exchange should increase to a maximum and then decrease to zero as the concentrations of fumarate and AMP-S are increased in fixed ratio, according to the equation

\[
R_{\text{AMP}} = \frac{k_+ k_0 [E_f]}{k_2 k_3 + k_6 k_4 + k_7 k_2 + k_{k_5 R_{\text{AMP}}}} \\
+ k_2 (k_7 + k_2) \left( 1 + \frac{K_{\text{AMP}}}{[\text{AMP}] [F]} \right) \frac{K_5}{[F]} + k_{k_7 R_{\text{AMP}}}
\]

where \( K_2 \) is the equilibrium constant for Reaction 2. Fumarate exchange, on the other hand, should obey simple saturation kinetics as the concentrations of AMP and AMP-S are increased at constant concentration of fumarate.

\[
R_F = \frac{\left( \frac{k_{k_7}}{k_1 + k_2} \right) [E_f]}{1 + \frac{K_5}{[F]} + \frac{K_{K_{\text{AMP}}}}{[F][AMP]}} = \frac{R_{\text{Fmax}}}{1 + \frac{K_5}{[F]} + \frac{K_{K_{\text{AMP}}}}{[F][AMP]}}
\]

where \( R_{\text{Fmax}} \) is the exchange rate when \([F] \gg K_5 \) and \([\text{AMP}] \gg K_{\text{AMP}} \). However, as pointed out for bi-bi reactions by Boyer and Silverstein (6), if a partially compulsory path occurs, it may be revealed by incomplete inhibition of the exchange rate of one substrate pair by the other pair. This is also the case for the uni-bi mechanism, the rate equations being

\[
R_{\text{AMP}} = \frac{k_2 (k_7 + k_0) [E_f]}{k_1 + k_2 + k_3} \frac{k_{k_5 (k_7 + k_2) (1 + \frac{K_5}{[\text{AMP}]} )}}{k_{k_5 (k_7 + k_2) + k_{k_5 R_{\text{AMP}}}}}
\]

and

\[
R_F = \frac{k_2 (k_7 + k_0) (1 + \frac{K_5}{[F]} )}{k_{k_5 (k_7 + k_2) + k_{k_5 R_{\text{AMP}}}}}
\]

These equations are simplified to Equations 3 and 4 by setting \( k_5, k_7, \) and \( K_5 \) equal to 0 and substituting \( K_{K_{\text{AMP}}} \) for \( K_7 K_3 \). The general form of Equations 5 and 6, for exchange of Substrate A at varying concentrations of Substrate B, is

\[
R_A = \frac{a + bB}{c + dB + e} \frac{1}{B}
\]

where \( a, b, c, d, \) and \( e \) are constants.

At low concentrations of \( B \), the equation is simply that of a rectangular hyperbola.

1. The equations for equilibrium exchange rates in uni-bi reactions used in this paper are modifications of those derived by I. A. Rose for aldolase (personal communication).
and the response of $R_A$ to increases of $B$ is due to the decrease of $e/B$. At high concentrations of $B$, the equation becomes

$$R_A = \frac{a}{c + \frac{e}{B}}$$

Thus the ultimate rate of $A$ exchange as $[B]$ increases is $b/d$, and the exchange of $A$ may be stimulated or depressed by high $[B]$ depending on whether $b/d$ is higher or lower than $a/c$. From Equation 5 for $R_{AMP}$, when $AMP > K_4$

$$R_{AMP} = \frac{a + bB}{c + dB}$$

As $k_4$ is diminished, $b/d$ approaches 0, but $a/c$ does not. Therefore, at appropriately low values of $k_4$, $(a/c) > (b/d) > 0$, and $R_{AMP}$ will be incompletely inhibited by increasing the concentration of fumarate. In the case of $R_F$ (Equation 6), assuming $k_4 < k_3$ and $F > K_2$

$$R_F = \frac{b - k_4[E_d]}{k_4 + k_4}$$

and

$$a = \frac{k_4[E_d]}{k_4 + k_4 \left[1 + \frac{K_4}{[F]}\right]}$$

Thus $(b/d) > (a/c)$, unless $k_3 > k_4$, and therefore $R_F$ will be stimulated by high concentrations of AMP.

The mechanism of Scheme 2 can therefore explain both the incomplete inhibition of AMP exchange by high fumarate concentration and the stimulation of fumarate exchange by high [AMP]. However, it cannot explain the gradual stimulation of AMP exchange by fumarate, at the highest range of [fumarate] and [AMP-S], and it is therefore necessary to postulate that another path comes into play under these conditions. At high concentrations, fumarate or AMP-S or both must combine with some form of the enzyme and speed up a reaction of the exchange. This complication obscures estimation of the fraction of the reaction proceeding by way of the lower path of Scheme 2. All that can be said is that it must be small.

Further support for an essentially ordered mechanism is provided by our observations that the presence of AMP increases or decreases the reactivity of the enzyme to various chemical agents, whereas fumarate has no effect unless AMP is also present. These results suggest that the way in which AMP enables fumarate to be attached is by inducing a conformational change in the enzyme by which the necessary binding groups are brought into position in the active center. Presumably AMP-S too would bind to the enzyme by its AMP moiety and thereby induce the movement of enzyme groups into position to interact with the succinate part of the molecule. That the succinyl portion of AMP-S plays a role in binding to the enzyme is indicated by the 8-fold higher dissociation constant of AMP as compared with AMP-S, and also by the fact that AMP is a less potent inhibitor of adenylosuccinate lyase than is the 6-thio analogue of AMP-S, which has been shown by Hampton (19) to be slowly split by the enzyme. Hampton also found that the esterification of the carboxyl groups of the thio analogue facilitates the base-catalyzed elimination, and has therefore proposed that the two carboxylic groups of AMP-S are neutralized in the $E$-AMP-S complex by positively charged groups of the protein.

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