Mechanism of Action of Amino Acid Transfer Ribonucleic Acid Ligases*

Robert B. Loftfield and Elizabeth Ann Eigner

From the Department of Biochemistry, School of Medicine, University of New Mexico, Albuquerque, New Mexico 87106

SUMMARY

The first reaction in protein biosynthesis has been presumed to be the interaction of ATP, amino acid, and enzyme (amino acid-activating enzyme, aminoacyl transfer RNA ligase, tRNA synthetase) to form an enzyme-bound aminoacyl adenylic anhydride. Such aminoacyl anhydrides could react forward with hydroxylamine or with tRNA or backward with [32P]-PPi. Experiments, primarily involving inhibitors, cast serious doubt on the existence of such a common intermediate in the enzyme-catalyzed formation of amino acid hydroxamate or [32P]-ATP from amino acid, ATP, and hydroxylamine, or [32P]-PPi, respectively. Studies with several enzymes indicate the probability of a general base-stimulated concerted reaction in which amino acid hydroxamate, AMP, and PPi are formed with no discrete intermediates. The esterification of tRNA resembles hydroxamate formation more than it resembles ATP-PPi exchange, strengthening the argument that the physiologically significant reaction does not involve an enzyme-bound aminoacyl adenylate. When tRNA is esterified in the absence of other bases, it is probable that one of the terminal ribose hydroxyls serves as a general base while the other is being acylated.

Since 1955 (1) it has been first postulated and then generally accepted that the first step in protein biosynthesis is the enzymatic interaction of ATP and amino acid to form enzyme-bound aminoacyl adenylate and free inorganic pyrophosphate ion. A second step, discrete from the first, consists of the transfer of the aminoacyl moiety to the terminal ribose unit of transfer RNA (2). In the first of these reactions, homologous amino acids tend to react like the corresponding natural substrate (3, 4), although the specificity is considerably greater than for any other comparable enzymatic or chemical reactions (5). On the other hand, the specificity with which the tRNA is esterified by a particular amino acid is several orders of magnitude greater. Some of this greater specificity has been accounted for experimentally (6, 7), but much of it is beyond present theoretical concepts.

These reactions have enormous interest for three reasons. The physical chemical problem of how close homologues can be distinguished so effectively, the potential role of these reactions in the control of metabolic processes, and the probability that the ultimate specificity of protein biosynthesis resides in the specificity of esterification of an appropriate tRNA makes it desirable to work out the details of these reactions. Therefore, we determined to study the mechanism of these two first steps. The preliminary evidence indicates that the basic concept of the obligatory formation of an intermediate aminoacyl adenylate is in doubt.

EXPERIMENTAL PROCEDURE

Materials—L-1-[14C] Amino acids were prepared by the Bucherer hydantoin method from Ba14CO3 (8, 9). All [14C]- and [12C]-amino acids were regularly assayed by column chromatography for impurities. All amino acids were prepared and used at specific activities between 20 and 30 mCi per mmole. Salt-free hydroxylamine was prepared by neutralization of methanolic hydroxylamine hydrochloride with methanolic sodium hydroxide followed by two distillations (10).

[32P] Pyrophosphate was prepared by pyrolysis of [32P] Na2 HPO4 followed by anion exchange column purification (9). Some preparations were purchased from New England Nuclear which, we understand, uses the same procedure. Imidazole, phenanthroline, ATP, collidine, lutidine, pyridine, amino acids, Tris, and inorganic compounds were commercial products of highest available purity. β,γ-[32P]-ATP was prepared by enzymatic incorporation of [32P]-PPi into ATP with the valine-activating enzyme (11). [32P]-ATP, [32P]-ATP, [32P]-ADP, and [32P]-AMP were purchased from Schwarz. tRNA was prepared by phenol extraction of Escherichia coli strain B cells (12) or purchased from Schwarz.

E. coli-activating enzyme specific for isoleucine, valine, leucine, and phenylalanine were prepared by minor modifications of the methods of Bergmann, Berg, and Dieckmann (13) and of Conway, Lansford, and Shive (14). The enzymes as finally

* This research was supported by Research Grant CA-08000 from the United States Public Health Service and by American Cancer Society Grant P-487.
eluted from a DEAE-cellulose column were protected with 60 mg/100 cc of reduced glutathione and assayed by [PP]-PP$_i$-ATP exchange, hydroxamate formation, and aminoacyl-tRNA formation. Each enzyme preparation was somewhat unstable, but lost little activity in storage at -15°. In each case the specific activity was comparable to but somewhat less than the maximum activity reported by the above workers. Each preparation was analyzed with gel electrophoretic techniques and appeared to be about 60 to 80% pure.

Methods—For [PP]-PP$_i$-ATP exchange, unless otherwise indicated, 5.0 mM concentrations of [PP]-PP$_i$ and ATP (Mg$^{2+}$ salt) were incubated with Tris buffer (pH 7.5, 0.1 n), appropriate amounts of amino acid and enzyme, 200 µg of bovine serum albumin per ml, and 1 mM MgCl$_2$ in a total volume of 200 µl. These are essentially the conditions described by Bergmann et al. (13). At least three aliquots of 50 µl were removed at time intervals up to 40 min. In our hands separation of ATP from PP$_i$ by absorption on charcoal (13) was found to be erratic. Therefore, we used the more reliable anion exchange paper method (5). The same technique was used to determine the rate of conversion of β,γ-[PP]-ATP to PP$_i$.

The hydroxamate assay was in general conducted with 10 mM ATP (Mg$^{2+}$), 2.5 mM NH$_3$OH (adjusted to pH 7.0 with HCl), and appropriate amounts of [³C]-amino acid and activating enzyme. Three or four aliquots were taken at intervals up to 40 min, and the formed [³C]-hydroxamate was separated from unreacted [³C]-amino acid on cation exchange paper (5, 10, 15).

Inhibition, or approach to equilibrium. We have, therefore, attempted to determine true initial rates by determining the extent of each reaction at no fewer than three or four intervals at times when the reaction was only partially completed. When destruction of substrate (ATP or tRNA) was a possibility, we also determined the extent of reaction at "infinite" time. Figs. 1, 2, and 3 are entirely typical experiments that show linearity over the initial phase of the reaction studied. It is to be noted that, on occasion, the intercept with the ordinate is below zero, possibly a consequence of an induction period or the formation of a transient intermediate (26).

Intercconversion of [³C]-AMP, [³C]-ADP, and [³C]-ATP (or the corresponding tritiated nucleotide) was followed by separation with thin layer exchange (polyethyleneimine medium) (16). An aliquot of the reaction mixture was placed on the thin layer chromatography origin; elution was begun with 25% methanol and followed by 1 M LiCl. AMP, ADP, and ATP separate cleanly and can be located by their quenching of the ultraviolet fluorescence of the medium. The nucleotide-containing areas are cut from the plastic support and assayed for radioactivity in a scintillation spectrophotometer. Estification of tRNA by homologous enzymes and amino acids was generally conducted by estimating the incorporation of a [³C]-amino acid into a trichloroacetic acid-insoluble precipitate of protein and tRNA according to methods described previously (17).

We have taken special care to determine initial reaction rates. A single measurement of reaction extent is always hazardous, especially when inhibition is being studied. A single time observation may obscure a very high zero time background, a progressive deterioration in the enzyme, product or substrate inhibition, or approach to equilibrium. We have, therefore, attempted to determine true initial rates by determining the extent of each reaction at no fewer than three or four intervals at times when the reaction was only partially completed. When destruction of substrate (ATP or tRNA) was a possibility, we also determined the extent of reaction at "infinite" time. Figs. 1, 2, and 3 are entirely typical experiments that show linearity over the initial phase of the reaction studied. It is to be noted that, on occasion, the intercept with the ordinate is not zero, indicating a significant background (Fig. 1) or an induction period.
leucine, and water to a total volume of 325 μl. Aliquots of 75 μl
were removed at 2, 4, 6, and 30 min. The protein and isoleucyl-
tRNA were precipitated and washed, and the incorporated radio-
activity was determined as described in the text.

FIG. 3. A typical experiment showing the rate of isoleucyl-
tRNA formation as a function of isoleucine concentration. Each
reaction mixture contained 0.1 mM Tris (pH 7.5), 10 mM ATP (Mg²⁺
salt), 40 μg of isoleucine enzyme solution, 5 mg of bovine serum
albumin, 30 μg of tRNA, the indicated concentration of [¹⁴C]-iso-
leucine, and water to a total volume of 325 μl. Aliquots of 75 μl
were removed at 2, 4, 6, and 30 min. The protein and isoleucyl-
tRNA were precipitated and washed, and the incorporated radio-
activity was determined as described in the text.

Exhaustion, substrate destruction, product inhibition, or enzyme
deterioration. In each case, however, and in the case of every
rate reported in this study, it was possible to determine a con-
stant initial rate. It should be noted in each of these figures
that the rate is constant even when the reaction rate is 90%
inhibited. This is evidence that the inhibition is not a conse-
quence of a progressive destruction of enzyme activity.

Arguments have been presented by others (18, 19) that per-
suasively support the superiority of the Eadie plot (20) (r plotted
against 1/S) over the Lineweaver-Burk plot (1/v plotted against
1/S) for the determination of enzymatic constants. We have
used only the Eadie plot.

RESULTS

It is widely assumed that for each of the naturally occurring
amino acids there is a corresponding activating enzyme (ammo-
noacyl-tRNA ligase, tRNA synthetase) that catalyzes the following
reactions:

\[
\text{AA} + \text{ATP} + E + \text{tRNA} \rightarrow \text{E(AMPA)} + \text{PP}_{\text{i}} + \text{AMP}
\]

Where AA is the generic term for α-amino acid, \(\text{AA} \sim \text{AMP}\) is
the anhydride in which the phosphoric residue of adenylate acid is
electronically bound to the carbonyl carbon of an amino acid,
\(\text{AA} \sim \text{tRNA}\) is an anhydride (or ester) between amino acids and
tRNA of relatively high reactivity, and \(\text{AA-NHOH}\) is amino
acid hydroxamates.

The formation of the enzyme aminoacyl adenylate complex
\((E(\text{AA} \sim \text{AMP}))\) is believed to be rate-determining. This
inference is supported by the linear dependence of each reac-
tion on enzyme concentration (13) and by the Michaelis-Menten
dependence of Reactions 1A (13), 1B (5), and 1C (5) on ATP
and amino acid concentration. Synthetic aminoacyl adenylnates
have been prepared (21) and, in the presence of the enzyme,
react with pyrophosphate ion to regenerate ATP (21) or with
tRNA to form the aminoacyl tRNA ester (22). It has been
noted qualitatively that high concentrations of pyrophosphate
ion suppress hydroxamate formation (23, 24) or, conversely,
that destruction of the pyrophosphate ion with a pyrophosphatase
stimulates hydroxamate production (25). It was the purpose
of this work to study the competition between Reactions 1A, 1B,
and 1C.

When one attempts to analyze quantitatively the inhibition
of Reaction 1A by hydroxylamine or other bases or the inhibition
of Reaction 1B by pyrophosphate ion, it is seen immediately that
the behavior of the activating enzymes cannot be adequately
described by the postulated reaction scheme. If hydroxylamine
inhibits the interaction of enzyme-bound aminoacyl adenylate
with \([32P]\)-pyrophosphate ion by itself reacting with the inter-
mediate according to Reaction 1B, it is obvious that 1 molecule
of amino acid hydroxamate and 1 molecule of adenyl acid should
result from the destruction of each enzyme-bound amino-
acyl adenylate that is made unavailable for reaction with \([32P]\)-
pyrophosphate ion. In other words, there should be something
of an equality between the number of moles of \([32P]\)-pyrophos-
phosphate ion that is inhibited from reacting with the aminoacyl
adenylate and the number of moles of hydroxylamine that react
with the aminoacyl adenylate to form hydroxamate and adenyl
cid. Tables I and II show that there is no such equality in the

TABLE I

<table>
<thead>
<tr>
<th>(a) 2.5 mM NH₄OH</th>
<th>(b) 0.74 mM imidazole</th>
<th>(c) 0.015 mM phenanthroline</th>
</tr>
</thead>
</table>

\[ \text{PP}_{\text{i}} \text{ to ATP exchange, no inhibitor added.} \]

<table>
<thead>
<tr>
<th>Same + inhibition</th>
<th>375</th>
<th>375</th>
<th>375</th>
</tr>
</thead>
</table>

\[ \text{[³⁴C]-AMP formed from [³⁴C]-ATP} \]

<table>
<thead>
<tr>
<th>\text{[³⁴C]-Valine converted to hydroxamate}</th>
<th>\text{0.2 mM PP}_{\text{i}} \text{ present.}</th>
<th>\text{0.2 mM PP}_{\text{i}} \text{ present.}</th>
</tr>
</thead>
<tbody>
<tr>
<td>\text{3.0}</td>
<td>&lt;1.0</td>
<td>&lt;1.0</td>
</tr>
</tbody>
</table>

\[ \text{Difference = amount of inhibition.} \]

<table>
<thead>
<tr>
<th>\text{No PP}_{\text{i}} \text{ present.}</th>
<th>\text{3.0}</th>
<th>&lt;1.0</th>
<th>&lt;1.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>\text{[³⁴C]-Valine converted to hydroxamate}</td>
<td>\text{0.2 mM PP}_{\text{i}} \text{ present.}</td>
<td>\text{0.2 mM PP}_{\text{i}} \text{ present.}</td>
<td></td>
</tr>
<tr>
<td>\text{3.0}</td>
<td>1.5</td>
<td>1.9</td>
<td></td>
</tr>
</tbody>
</table>

\[ \text{No PP}_{\text{i}} \text{ present.} \]

| \text{3.0} | 1.5 |

\[ \text{Same as a, but with 0.015 mM o-phenanthroline and 1.25 mM PP}_{\text{i}}. \]

\[ \text{Same as e, but with 1.0 mM ATP and 0.8 mM [³⁴C]-valine.} \]

\[ \text{With 5 mM ATP, 0.1 mM PP}_{\text{i}}, \text{0.5 mM valine.} \]

\[ \text{Same as a, but with 2.5 mM NH₄OH.} \]

\[ \text{Same as a, but with 0.74 mM imidazole and 3 mM PP}_{\text{i}}. \]

\[ \text{Same as a, but with 0.015 mM o-phenanthroline and 1.25 mM PP}_{\text{i}}. \]

\[ \text{Same as e, but with 1.0 mM ATP and 0.8 mM [³⁴C]-valine.} \]
case of E. coli valine-activating enzyme and isoleucine-activating enzyme. For instance, in the absence of hydroxylamine, 375 μmole of [32P]pyrophosphate ion are incorporated into ATP per min per ml of valine enzyme solution. The inclusion of 2.5 mM hydroxylamine, reducing the rate of ATP-PPi exchange 97%, prevents some 362 μmole of [32P]pyrophosphate ion from reacting. However, this amount of enzyme solution is capable of producing only 2.0 to 3.0 μmole of valine hydroxamate per min depending on whether pyrophosphate ion is present. It is possible that hydroxylamine reacts with the valyl adenylate to form some unstable product (an O-aminoacyl hydroxylamine?) (26) that may or may not yield a stable N-valyl hydroxamic acid. Even if this should be the case, there ought to be 1 molecule of adenylic acid formed for each ion of [32P]pyrophosphate ion that fails to react with the postulated valyl adenylate. In fact, Table I shows an almost exact equivalence between hydroxamate and AMP formed, each being formed only about 0.01 as rapidly as necessary to account for the inhibition of ATP-PPi exchange. Table I shows two other nucleophilic substances to be potent inhibitors of ATP-PPi exchange. Both imidazole and phenanthroline might be expected to react with valyl adenylate to form unstable valyl derivatives that might hydrolyze without being detected. However, here again, there should be AMP production commensurate with the extent of inhibition of incorporation of [32P]pyrophosphate ion into ATP. Within the limits of detection, no AMP is formed even when the exchange reaction has been inhibited 50 to 90%.

Similar evidence is presented in Table III. If the proposed reaction scheme is correct, the formation of hydroxamates (Reaction I) should be inhibited by pyrophosphate ion, one [32P]pyrophosphate ion entering ATP for every molecule of isoleucyl adenylate made unavailable for hydroxamate synthesis. In this case, the formation of 4.2 μmole per min of hydroxamate has been prevented under conditions which permit the formation of only 0.12 μmole per min of [32P]-ATP.

These data conclusively establish that the inhibition of ATP-PPi exchange by various nucleophiles or the inhibition of hydroxamate formation by pyrophosphate ion cannot be a consequence of competition between the several reagents for an enzyme-bound aminoaeryl adenylate. If an enzyme-bound aminoaeryl adenylate is the intermediate in the ATP-PPi exchange reaction, it follows that the effect of hydroxylamine or other nucleophiles must precede the formation of the aminoaeryl adenylate. Reaction Scheme 1 does not predict any effect of hydroxylamine on the forward reaction to form enzyme-bound aminoaeryl adenylate and free pyrophosphate ion. When β,γ-[32P]-ATP is reacted with valine and the valine enzyme, we observe that 2.5 mM hydroxylamine inhibits the formation and release of [32P]pyrophosphate ion by 97%, essentially the extent to which 2.5 mM hydroxylamine inhibits the incorporation of [32P]pyrophosphate ion into ATP.

The data presented in Tables I, II, and III are inconsistent with the postulate that enzyme-bound aminoaeryl adenylate are obligatory intermediates in the formation of hydroxamates. The evidence from these inhibition experiments requires abandonment of the scheme for formation of hydroxamates, modification of the scheme for ATP-PPi exchange, and reconsideration of the mechanism by which tRNA is esterified by specific amino acids. It has been noted by Takets and Pogell (27) and by Atkinson (28) that the effect of inhibitors on enzyme activities can often be profitably expressed in the form of the Hill (29) equation,

$$\log (v/v_i - 1/v_o) = C + n \log[I]$$

where $v$ is the reaction rate, $v_o$ is the rate of the reaction under identical conditions except that no inhibitor is present, $C$ is a constant term which may or may not be a function of substrate concentration, $[I]$ is the concentration of inhibitor, and $n$ is the number of molecules of inhibitor cooperatively reacting with each molecule of enzyme to cause total inactivation. When $\log (1/v - 1/v_o)$ is plotted against $\log[I]$, the slope of the resulting straight line is $n$. In all cases in which we have been sought, we have found the following relationships with the E. coli-activating enzymes specific for valine, isoleucine, leucine, and phenylalanine.

1. Hill plots show that ATP-PPi exchange appears to be inhibited by 1 molecule of phenanthrolide ($K_I = 10^{-4}$ to $10^{-5}$ M) (Fig. 4) or cooperatively by at least 2 molecules of hydroxylamine or by 2 molecules of imidazole or 1 molecule each of hydroxylamine and of imidazole ($K_I = 0.1$ to $1.0$ M) (Fig. 5).

2. Esterification of tRNA is noncompetitively inhibited by 1 molecule of phenanthrolide ($K_I = 0.018$ M) (Fig. 6) or by 1 molecule of PPi ($K_I = 10^{-4}$ to $10^{-5}$ M) (Figs. 7 and 8) while esterification of tRNA is generally stimulated by the presence of nonfunctional general bases such as imidazole, ammonia, collidine, lutidine, and hydroxylamine (30).

3. The rate of formation of amino acid hydroxamates is second order in hydroxylamine when no other bases are present.

### Table II

**Balance sheet for extent of inhibition of ATP-PPi exchange and appearance of isoleucine hydroxamate and AMP**

| Conditions: 10^{-4} M PPi, 2 x 10^{-4} M ATP, 5 x 10^{-3} M isoleucine, 0.1 mM Tris buffer (pH 7.5), 0.5 mg per ml of albumin, and 1 μl of Escherichia coli isoleucine enzyme incubated at 25°. Aliquots were removed at 5-min intervals and assayed as described in the text. Units are micromoles converted per min per ml of isoleucine enzyme solution. |
| PPi to ATP exchange, no inhibitor | Same + 2.8 M NH_4OH (pH 7.5) | Difference = amount of inhibition |
| 133 | 1.6 | 131.4 |

**Table III**

**Balance sheet for inhibition of isoleucine hydroxamate formation as inhibited by PPi**

| Conditions: each reaction vessel contained 0.10 mM isoleucine-14C, 2.5 mM ATP (Mg2+), 5.6 M NH_4OH (pH 7.5), 0.1 M Tris (pH 7.5), and 6 μl of Escherichia coli isoleucine enzyme. The second and third reaction vessels contained 0.1 mM PPi. The third reaction mixture was identical with the second except that the PPi was radioactive and conversion to [32P]-ATP was determined. The reactions were carried out at 25° and aliquots were removed at 0, 5, 10, 15 min. Units are micromoles converted per min per ml of isoleucine enzyme solution. |

| [14C] Isoleucine hydroxamate formation |
| No PPi | Plus 0.1 mM PPi | Difference = extent of inhibition |
| 5.45 | 1.20 | 4.25 |

| ATP-PPi conversion with 0.1 mM [32P]-PPi | 0.12 |
Amino Acid tRNA Ligases Vol. 244, No. 7

I

0.01 0.02 0.03 MOLAR (PHENANTHROLINE)

FIG. 4. A typical experiment showing inhibition of valine-catalyzed ATP-PP\(_{i}\) exchange by 3-phenanthroline. Conditions were as described in the text except that phenanthroline was added in the indicated amounts. The inset is essentially a Hill plot, the slope of 1.0 indicating that a single molecule of 3-phenanthroline may be responsible for inactivating 1 molecule of enzyme.

FIG. 5. Typical experiments showing the inhibitory effects of hydroxylamine or imidazole on the valine-catalyzed ATP-PP\(_{i}\) exchange. The inset is a Hill plot whose slope of 2.0 for each inhibitor suggests a strongly cooperative effect by 2 molecules of the base in inactivating the enzyme.

If a monofunctional general base such as imidazole is present, the rate of hydroxamate formation is first order in hydroxylamine (90). At all concentrations of hydroxylamine, 1 molecule of PP\(_{i}\) inhibits the hydroxamate-forming ability of the enzyme (Fig. 9).

All of these observations are consistent with the premise that a divalent cation such as ferrous (or cadmium, zinc, or manganous) ion is involved in the step or steps which determine the rate of tRNA esterification, ATP-PP\(_{i}\) exchange, or hydroxamate formation. The hydroxamate-forming reaction is most easily visualized as a concerted reaction in which 2 hydroxylamine molecules (or 1 hydroxylamine molecule and another molecule of a general base) are bound to the metal. In a single reaction a proton is transferred from the reacting hydroxylamine molecule to the general base, a bond is formed between the carbonyl carbon and the hydroxylamine molecule, the hydroxyl oxygen of the amino acid is transferred to the \(\alpha\)-phosphorus of ATP, and the \(\beta,\gamma\)-phosphorus group of ATP is displaced as free PP\(_{i}\). This scheme may be visualized in Fig. 10 by imagining the left-hand hydroxyl of tRNA as NH\(_{2}\)OH and the right-hand hydroxyl of tRNA as the general base (in the case of hydroxamate formation, the reaction is, of course, not reversible).

An entirely analogous series of reactions occurs when tRNA

FIG. 6. A typical experiment showing noncompetitive inhibition of tRNA esterification by 3-phenanthroline. The conditions were as described in the text. If the concentration of 3-phenanthroline is varied while other conditions are held constant, a Hill plot indicates that 1 molecule of 3-phenanthroline is reacting with 1 molecule of enzyme. (The \(K_m\) for tRNA\(_{Val}\) is about one-tenth of normal because of the inclusion of 10% ethanol for the purpose of solubilizing the phenanthroline (17).)

FIG. 7. Typical experiments showing noncompetitive inhibition of tRNA esterification of PP\(_{i}\). Conditions were as described in the text with tRNA concentrations varying from 0.3 mg per ml to 9.8 mg per ml. The concentration of PP\(_{i}\) was 0.0, 0.17, or 0.29 mm.
reacts with amino acid and ATP. The 2'-OH and 3'-OH residues of the terminal adenosine form complexes with the metal. One of the hydroxyl groups is the nucleophile that attacks the carbonyl carbon while the other hydroxyl temporarily accepts the released proton, thus serving as a general base. If some other, better general base is available the reaction is accelerated (30). In other respects the reaction is similar to the hydroxamate forming reaction (Fig. 10).

The ATP-PP\textsubscript{i} reaction can also be written in an analogous fashion, 1 ion of pyrophosphate binding to the metal ion, 1 of the oxygen atoms of the pyrophosphate serving as a nucleophile displacing the hydroxyl radical from the carbonyl carbon, and so forth. The products of this reaction would be an aminoacyl pyrophosphate, AMP, and PP\textsubscript{i}. Since the resultant aminoacyl pyrophosphate might be expected to have a very high free energy of hydrolysis, the reaction would be easily reversible and \([^{32}\text{P}]\text{PP}_i\) would be incorporated into ATP. This scheme might also provide for the incorporation of \([^{14}\text{C}]\text{AMP}\) into ATP under conditions in which ATP-PP\textsubscript{i} exchange is occurring. Although such an incorporation was not observed with the tryptophan enzyme (25), we sought such a reaction with the purest preparations of valine-activating enzyme (including a gift from Dr. Bruce Jacobsen of Oak Ridge National Laboratories). The rate of incorporation of AMP into ATP never exceeded 1% of the rate of incorporation of PP\textsubscript{i} into ATP. Although one may interpret this result as a consequence of a very slow dissociation of AMP from the enzyme, it is probably more realistic to consider alternative schemes.

The most likely alternative (Fig. 11) is that, in the absence of other bases, the amino acid does form an aminoacyl adenylate with the release of PP\textsubscript{i}. It would be anticipated that in the transition state the charge concentration on the incipient pyrophosphate ion would be so high that there would be a need to dissipate the charge. This could be done most easily by coordination of the negative oxygens of the putative metal ion. If the metal ion were already coordinated with bases such as phenanthroline or hydroxylamine, it would be inaccessible to the developing pyrophosphate ion and, correspondingly, the measurable ATP PP\textsubscript{i} exchange or \([^{32}\text{P}]\text{PP}_i\) release would be inhibited.

Fig. 10. A schematic representation indicating a possible concerted reaction leading to esterified tRNA, AMP, and PP\textsubscript{i}. The unknown metal, \(M^{++}\), is shown as being tightly bound to the enzyme through a sulfur. One hydroxyl of the tRNA serves as a general base, accepting 1 proton from the other hydroxyl. Other general bases may be more effective (30). If bases like NH\textsubscript{2}OH are substituted for the HO—C—C—OH residue of tRNA, the scheme is applicable to the general base-catalyzed formation of hydroxamates except that the reaction is not reversible.
**FIG. 11.** A schematic representation of the enzyme-catalyzed conversion of amino acid and ATP to enzyme-bound aminoacyl adenylate and free PPi. The function of the unknown metal, $M^{++}$, is to dissipate partially the negative charge on the incipient pyrophosphate ion in the transition state.

**Fig. 12.** The dependence of isoleucine-catalyzed ATP-PPi exchange on PPi concentration. Conditions were as described in the text except that [ATP] was held constant at 10^{-4} M while [PPi]-PPi concentration was varied from 10^{-4} M to 1.25 \times 10^{-3} M. In the reaction with 7 \times 10^{-4} M PPi, 37\% of the ATP was converted to a radioactive form, by far the highest conversion included in any of the experiments reported in this paper. The concentration of MgCl$_2$ was constant at 0.01 M and thus exceeded the combined concentrations of ATP and PPi in all experiments except the two with highest PPi concentration. Thus the inhibition cannot be ascribed to the formation of a Mg-PPi complex that makes Mg$^{2+}$ inaccessible to the enzyme and ATP. No precipitate of magnesium pyrophosphate was observed.

Schweet and Allen (31) and we (5) have previously reported that the rate of ATP-PPi exchange varies with PPi concentration according to Michaelis-Menten kinetics, the $K_m$ for PPi being in the range of 10^{-4} M. In each case the concentration of ATP had been held constant at about 5 mM. It is clear from Fig. 11 that any bidentate nucleophile would be a good inhibitor of the synthesis of enzyme-bound aminoacyl adenylate and correspondingly the incorporation of [PPi]-PPi into ATP. Just as 15 mM phenanthroline inhibits ATP-PPi exchange about 50\% we would expect comparable concentrations of PPi to be strongly inhibitory. Fig. 12 shows that 3 mM PPi inhibits the ATP-PPi exchange about 90\%. A Hill plot of the data shows a slope of 1.0 for the five highest concentrations of PPi, indicating again that 1 ion of PPi inactivates 1 molecule of enzyme. As in the other cases, this inhibition must occur at a point preceding the formation and release of PPi and must involve binding of PPi to the active site of the enzyme. These concentrations of PPi are inadequate to reverse the equilibrium favoring AA ~ tRNA and the increased ionic strength would not be expected to slow the reaction significantly (17). Moreover, the $K_m$ for ATP determined at constant PPi concentrations is found to vary from 3 \times 10^{-4} M to 1.8 \times 10^{-3} M depending on whether the PPi concentration is 2 \times 10^{-4} M or 2 \times 10^{-3} M respectively. The kinetics is partially competitive, suggesting that part of the association of ATP with enzyme is due to coordination with the metal at the active center.

One wonders about the effect of tRNA as a potential inhibitor of ATP-PPi exchange if the two hydroxyls form complexes with the metal. It has already been noted by us (7) and others (6) that tRNA may induce conformational changes so great as to obscure relatively small effects due to the adenosine hydroxyls. With these four enzymes the addition of tRNA has no significant effect on the rate of ATP-PPi exchange. Although the $K_m$ values for tRNA (in the AA ~ tRNA-forming reaction) are generally in the range of 10^{-7} to 10^{-8} M, and although these are probably true dissociation constants, it will be realized that almost all of this strong binding can result from interaction of some 80 nucleotides with the enzyme. Very little of the binding need involve the terminal adenosine and the metal. This is clear from early experiments in which oxidized (nonreceptive) tRNA affected enzyme function just as native tRNA (7). Further proof comes from the above-noted fact that the esterification of tRNA is noncompetitively inhibited by PPi and phenanthroline (Figs. 6 and 7). In developing the equations for noncompetitive inhibition, it is assumed that the binding of inhibitor to free enzyme is exactly the same as the binding of inhibitor to enzyme-substrate complex. A direct corollary is that the metal ion involved in ATP-PPi exchange is equally accessible for coordination with the developing PPi regardless of the presence of tRNA. Although the metal ion is essential for the execution of the tRNA esterification reaction, it forms complexes with the adenosine hydroxyls only a small part of the time.

Efforts have been made to remove the metal from the enzyme. Although ferrous ion appears to be present in our purest enzymes, 5-hour dialysis at 0° against 10 mM phenanthroline and glutathione does not inactivate the valine enzyme. We have been
unsuccessful in the only efforts that we have made to show metal ion activation of the enzymes.

**DISCUSSION**

All known amino acid tRNA ligases appear to catalyze the formation of aminoacyl tRNA with the concurrent splitting of ATP into AMP and PPi. The acetothiokinase-catalyzed synthesis of acetyl-CoA involves the same cleavage of ATP. For this latter reaction, Webster (32) has shown unequivocally that, in addition to Mg$^{2+}$, another doubly charged cation such as Fe$^{2+}$ or Cu$^{2+}$ is required. The conversion of acetyl adenylate to acetyl-CoA requires the heavy metal but not magnesium as previously noted by Berg (33). In this well studied reaction, it has been generally accepted that an enzyme-bound acetyl adenylate is the intermediate just as an enzyme-bound aminoacyl adenylate is considered a probable intermediate in the activation of amino acids. Jencks (34) has reviewed the evidence for and against the acetyl adenylate intermediate and concludes that, if acyl adenylates are intermediates, they must be bound to the enzyme by ill-defined but extremely strong forces which effectively deactivate the intermediate. Because the concept of an enzyme-bound chemically unreactive acyl adenylate is not completely satisfactory, Jencks (34) considers briefly the possibility of a concerted reaction such as we have proposed above for the esterification of tRNA. By implication, acetyl adenylate could be formed or utilized but not in the direct pathway from acetate to acetyl-CoA.

The analogy to amino acid activation is obvious. Although Webster was able to remove the metal ion by dialysis or treatment with ion exchange resins, we have been able to show probable metal participation only with inhibitors. Webster finds that the heavier metal is involved in both of the presumed sequential reactions while Mg$^{2+}$ is only involved in reactions in which an AMP-PPi bond is being made or broken; our reaction schemes show the participation of the heavy metal in all reactions. If we had begun with an aminoacyl adenylate it is quite possible that Mg$^{2+}$ would not have been required to yield aminoacyl tRNA. Finally, the chemical structure of the receptor end of CoA is analogous to the structure of the receptor nucleotide of tRNA, the sulphydryl and amino corresponding to the two hydroxyls.

The evidence for enzyme-bound adenylates as intermediates in amino acid activation is generally similar to that for the participation of acetyl adenylate in the formation of acetyl-CoA. Concern has been expressed about the probably very high reactivity of the aminoacyl adenylates and how this is controlled by some sort of tight association with the enzyme.

Stulberg and Novelli (35) have discussed thoroughly all of the evidence for intermediate enzyme-bound aminoacyl adenylates. They conclude that, as of 1962, all evidence is consistent with Reaction Scheme 1, but that a concerted reaction such as outlined in Fig. 10 is not to be ruled out.

The biggest distinction between the aminoacyl-tRNA ligases that we have studied and Webster’s acetothiokinase appears to be in the ease of removal of the metal. We have tried to show this in Figs. 10 and 11 by indicating the metal is bound to the enzyme through sulfur. Almost all ligases reported to date are extremely sensitive to sulphydryl reagents or oxygen, from which we infer that sulfur may help to bind the cation.

A comparison of Figs. 10 and 11 shows a striking modification of the arrangement of the atoms about the reactive center. The presence of tRNA or even simple nucleophiles such as hydroxylamine must induce conformational changes so great that entirely different enzymatic activity might result. Norris and Berg (11) observed that the presence or absence of tRNA$^{12}$ determined whether the isoleucine enzyme would hydrolyze valyl adenylate or not. We (7) have noted substantial changes in the rates of formation of several hydroxamates depending on whether tRNA is present. Most dramatically, several investigators, the first being Mitra and Mehler (36), have reported aminoacyl tRNA ligases that are completely devoid of ATP-PPi exchange or hydroxamate-forming ability in the absence of the appropriate tRNA.

If there is a formation of a distinct E(AA$\sim$ AMP) as an intermediate between free AA and AA$\sim$ tRNA, the separate steps must individually be at least as fast as the over-all reaction. To our knowledge, only Berg (37) has specifically considered this problem and his methionine-activating enzyme meets the test. All other reported experiments in which previously formed aminoacyl adenylates are reacted with tRNA involve periods of reaction such that a very slow reaction could easily go to completion.

It must be reemphasized that our data are initial rates of reaction, determined at three or four time intervals under conditions in which even the fastest reaction was linear and proceeded no further than 20%. In every case one sample was permitted to run for 10 or 20 times the usual reaction time to establish that the final extent of conversion was independent of the concentration of pyrophosphate. Thus we are confident that we are dealing with initial rates and not a complex of approach to equilibrium, changed equilibrium, progressive denaturation of enzyme, or exhaustion of substrate.

We are left with a strong argument that a polyvalent cation is involved in the binding and subsequent reaction of pyrophosphate, hydroxylamine, imidazole, and other simple bases, whereas only the reaction, but not the binding, of tRNA depends on the cationic center. It seems probable that a concerted reaction involving a general base (possibly a hydroxyl from the tRNA) provides the mechanism for esterification of tRNA or for hydroxamate formation while a quite different reaction pattern involving the intermediate formation of E(AA$\sim$ AMP) accounts for ATP-PPi exchange.

**REFERENCES**

