ENZYMATIC CARBOXYL ACTIVATION OF AMINO ACIDS*

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(Received for publication, June 3, 1955)

Previous work in this laboratory (1-3) had revealed that the incorporation of C\textsuperscript{14}-labeled amino acids into the protein of rat liver microsome fraction was dependent upon ATP\textsuperscript{1} and enzymatic components of the soluble protein fraction. Part of this enzymatic requirement was accounted for by enzymes which would generate ATP from a precursor such as phosphocreatine, phosphopyruvate, or phosphoglycerate. It was apparent, however, that, after fortification of the incorporation system with the precursors and the appropriate ATP generating enzymes, heat-labile, non-dialyzable components of the soluble fraction were still required.

It therefore seemed reasonable to subject to experimental test the possibility that a mechanism for amino acid activation by ATP resides in this soluble protein fraction. Preliminary results of such a study (4) revealed that the dialyzed soluble protein fraction of rat liver catalyzes an exchange of PP\textsubscript{32} with ATP which is enhanced several fold by the addition of a group of pure L-amino acids. The microsome fraction also catalyzes a PP\textsubscript{32} exchange which is not, however, influenced by amino acids. It was found, furthermore, that AMP fails both to inhibit the amino acid-dependent exchange and to exchange with ATP (by using C\textsuperscript{14}-labeled AMP). These results suggested that the amino acids were being activated as an amino acyl \sim AMP compound. This possibility was given further support by the finding that \alpha-amino hydroxamic acids are formed in the presence of high hydroxylamine concentrations, with concomitant loss of ATP. Since the amino acids did not cause a net splitting of ATP unless hydroxylamine was present, it was proposed that the amino acyl \sim AMP is bound on the enzyme surface and dissociates from the enzyme to only a small extent if at all. The L-amino acid effect on exchange and on hydroxamic acid formation was additive with different amino acids, and D-amino acids were inert in the system.

* This is Publication No. 856 of the Cancer Commission of Harvard University.
† Scholar in Cancer Research of the American Cancer Society.

\begin{align*}
\text{AMP and ATP} &= \text{adenosine mono- and triphosphate; } P = \text{inorganic orthophosphate; } PP = \text{inorganic pyrophosphate; } CTP, UTP, ITP = \text{cytidine, uridine, and inosine triphosphate; } GDP \text{ and GTP} = \text{guanosine di- and triphosphate; Tris} = \text{tris-(hydroxymethyl)aminomethane.}
\end{align*}
These findings suggested a hypothesis for amino acid activation in animal tissue (4), which has since received further support by partial purification of the system and a more detailed analysis of its properties. The present paper deals with these extensions of the earlier work.

EXPERIMENTAL

Enzyme Preparations—The livers of young (80 to 100 gm.) Wistar strain rats were used throughout. All operations were performed at 0°. 5 gm. of liver were homogenized with 7 ml. of 0.05 M KCl and, after this homogenate was diluted with 1 or 2 volumes of 0.05 M KCl, it was spun at 105,000 × g for 50 minutes in a Spinco preparative ultracentrifuge.

The soluble protein fraction of rat liver was drawn off with a syringe and the pH brought to 5.3 to 5.1 by the dropwise addition of 0.1 N HCl with constant stirring. The resultant precipitate was packed by centrifuging at 10,000 × g for 5 minutes. For certain experiments in which it was desirable to have minimal base-line exchange or hydroxamic acid formation, the soluble protein fraction was first diluted 6-fold with 0.05 M KCl and subjected to the same isoelectric precipitation, and the precipitate was washed once with a smaller volume of KCl. In all cases the precipitate was finally resuspended by homogenization in 0.1 M Tris buffer, pH 7.6 (to give a final protein concentration of about 30 mg. per ml.), re-centrifuged at 15,000 × g, and the undissolved material discarded. Protein concentration was determined turbidimetrically with trichloroacetic acid. (0.5 to 3 mg. of protein in 7.0 ml. of 3 per cent trichloroacetic acid was mixed thoroughly and read at 540 mμ in a Coleman spectrophotometer 30 seconds later. Standard calibration was carried out with known quantities of soluble protein fraction protein.) The preparation is referred to throughout as the pH 5 enzyme.

The fraction active for methionine hydroxamic acid formation was derived from the soluble protein fraction by treatment with saturated ammonium sulfate solution. The fraction precipitating between 35 and 45 per cent saturation was collected by centrifugation, resuspended in 0.005 M phosphate, pH 7.0, to give a final protein concentration of about 30 mg. per ml., and dialyzed against 200 times the volume of the same concentration of phosphate for 18 hours.

For the incorporation of C14-amino acids into protein in the anaerobic cell-free system, rat liver was homogenized in a sucrose medium (0.35 M sucrose, 0.035 M K2CO3, 0.025 M KCl, and 0.004 M MgCl2) under the conditions described previously (2). The homogenate was centrifuged for 10 minutes at 15,000 × g, and the supernatant fluid containing microsomes and the soluble cell fraction was used without further fractionation.

Materials—P32 was obtained from the Oak Ridge National Laboratories.
PP\textsuperscript{32} was prepared by pyrolysis of P\textsubscript{32} and contained less than 17 per cent of P\textsubscript{32}. (The pH 5 enzyme does not catalyze the P-ATP exchange.)

ATP, CTP, and UTP were obtained from the Pabst Laboratories. The Sigma Chemical Company supplied crystalline ATP, ITP, GDP, and GTP. Paper electrophoresis of this sample of GTP indicated the presence of considerable GDP.

Hydroxylamine was prepared salt-free from the hydrochloride by the method of Beinert et al. (5) and stored frozen.

Amino acids used in these studies were usually products from the Nutritional Biochemicals Corporation or the Schwarz Laboratories, and the purity of all had been checked by paper chromatography. Dr. Jesse Greenstein's group kindly supplied pure L-alanine, L-serine, L-isoleucine, and L-valine. The following twelve amino acids were used throughout: leucine, isoleucine, valine, glycine, threonine, histidine, phenylalanine, tryptophan, serine, alanine, arginine, and lysine. Glutamic and aspartic acids were omitted because of the activation of other than \(\alpha\)-carboxyl groups, proline because of its possible conversion to glutamic acid, cysteine because of its complicating sulfhydryl effect, tyrosine because of its low solubility, and methionine for reasons which become apparent below.

The hydroxamic acids of leucine, glycine, alanine, lysine, valine, and isoleucine were generously supplied by J. D. Gregory and S. Genuth. Methionine hydroxamic acid was prepared by the method of Safir and Williams (6) from methionine isopropyl ester, a gift of Dr. Max Brenner. Since paper chromatography or paper electrophoresis of these amino hydroxamic acids showed the presence of small amounts of impurities, particularly the corresponding amino acid, they are being purified further to provide exact standards.

**Exchange Studies**—The pH 5 enzyme was incubated for 8 minutes at 37\(^\circ\) in a volume of 1.0 ml. with 100 \(\mu\)moles of Tris buffer, pH 7.6, 5 \(\mu\)moles of ATP, 1 \(\mu\)mole of PP\textsuperscript{32}, pH 7.5, containing 100,000 to 200,000 c.p.m., 2 \(\mu\)moles of MgCl\(_2\), and amino acids as indicated. The low Mg\textsuperscript{++}:ATP ratio prevents enzymatic PP hydrolysis during the incubation. The reaction was stopped by adding 0.3 ml. of 25 per cent trichloroacetic acid, and the ATP and PP were separated and determined by charcoal adsorption according to the method of Crane and Lipmann (7). Under these conditions neither ATP nor PP was hydrolyzed. The results are expressed arbitrarily as per cent exchange, which is calculated as follows:

\[
\text{C.p.m. per \(\mu\)mole ATP} / \text{Total c.p.m. per \(\mu\)mole (ATP + PP)} \times 100
\]

This gives the specific activity of the ATP as per cent of the value which would be obtained at equilibrium.

**Hydroxamic Acid Formation**—The pH 5 enzyme was incubated at 37\(^\circ\)
for 50 minutes in a final volume of 2.0 ml. with 20.0 μmoles of the monomagnesium, dipotassium salt of ATP (except in experiments in which the Mg:ATP ratio was varied), about 2.4 mmoles of salt-free hydroxylamine, and amino acids according to the plan of the experiment. Hydroxamic acid was measured directly on a 1.0 ml. aliquot of the reaction mixture by adding 3 ml. of a solution of 10 per cent FeCl₃, 5 per cent trichloroacetic acid, and 3 N HCl, with use, as an internal standard, of an equimolar mixture of the first six amino hydroxamic acids mentioned above, or when appropriate, the specific amino hydroxamic acid. At the final pH of the FeCl₃ color development (about 1.0), the amino hydroxamic acids so far tested vary in color yield by no more than 20 per cent, most of them by less than that. After inactivating the enzyme in the remaining 1.0 ml. of incubation medium by placing the tubes in boiling water for 2 minutes, inorganic orthophosphate was determined by the method of Fiske and Subbarow on a 0.1 ml. aliquot, and was compared to a zero time control. PP was determined on a similar aliquot after ATP had been adsorbed on charcoal by the method of Flynn et al. (8).

Paper Chromatography of Reaction Products—Secondary butanol-formic acid-water in the ratio 75:15:10 and Whatman No. 3 filter paper were used for demonstrating amino hydroxamic acids. Genuth and Gregory had previously found this system to give good separation of these substances. Descending chromatograms were run for 12 to 18 hours and then sprayed with acidic FeCl₃ solution. Internal standards of known amino hydroxamic acids were used. The samples were prepared for chromatography by placing the incubation mixture in boiling water for 2 minutes, removing the protein by centrifugation, evaporating the supernatant solution to dryness, and desiccating for 24 hours in vacuo over concentrated H₂SO₄ to remove NH₂OH. The residue was redissolved in water and placed on paper.

Results

General Characteristics of Amino Hydroxamic Acid-Forming System—The activity of the pH 5 enzyme varied from day to day but usually corresponded to 0.4 μmole of hydroxamic acid formed per mg. of protein per hour at 37°C. The test was made with 10 μmoles of MgK₂ ATP, 1.2 mmoles of NH₂OH (pH 7.5), and 2 μmoles each of twelve amino acids, in a final volume of 1 ml. The pH 5 enzyme is about 5 times as active as the original soluble protein fraction. There was linear increase in hydroxamic acid formed with increasing Mg ATP concentrations up to 7 μmoles per ml., with maximal response at 10 μmoles. Fig. 1 shows the accumulation of hydroxamic acid with time and indicates that the reaction proceeds

Genuth, S., and Gregory, J. D., personal communication.
linearly for about 50 minutes. Fig. 2 relates hydroxamic acid appearance and NH$_2$OH concentration.

Although the enzymatic activity of the original soluble protein fraction of rat liver is rapidly lost at $-10^\circ$, the pH 5 enzyme can be stored frozen with little loss in activity. All enzymatic activity is lost upon heating at $50^\circ$ for 3 minutes. The stability properties of the system apply to the PP-ATP exchange reaction as well. The base-line exchange and hydrox-

![Fig. 1](http://www.jbc.org/)

**Fig. 1.** Time curve for hydroxamic acid formation. 7.8 mg. of pH 5 enzyme were incubated with 1.2 mmoles of NH$_2$OH, 10 μmoles of MgK$_2$ ATP, and 2 μmoles of each of twelve amino acids in a volume of 1 ml. at 37°. O, without amino acids; ●, with amino acids.

![Fig. 2](http://www.jbc.org/)

**Fig. 2.** Relation between hydroxamic acid formation and hydroxylamine concentration. 11.6 mg. of pH 5 enzyme were incubated at 37° for 60 minutes with 10 μmoles of MgK$_2$ ATP, 2 μmoles of each of twelve amino acids, and varying amounts of NH$_2$OH, in a volume of 1 ml. O, without amino acids; ●, with amino acids.

Stoichiometry of Pyrophosphate and Amino Hydroxamic Acid Formation—

It is possible to show good stoichiometry for hydroxamic acid formed, labile phosphate lost, and orthophosphate or pyrophosphate accumulated. Because of the high pyrophosphatase activity of the preparation, at optimal magnesium concentrations all pyrophosphate formed is converted to orthophosphate, and therefore 2 equivalents of phosphate are formed for every mole of hydroxamic acid formed. However, by adding fluoride or, as is illustrated in Fig. 3, reducing the Mg$^{++}$ concentration, pyrophosphatase can be completely inhibited, while the activation enzymes retain
some activity. Under these conditions, pyrophosphate accumulates in an amount equal to the amount of hydroxamic acid formed. At higher Mg\(^{++}\):ATP ratios, it can be seen that the orthophosphate divided by 2 plus the pyrophosphate equals the hydroxamic acid formed. Thus it was established that pyrophosphate is indeed the product of the reaction.

Reaction in Absence of Acceptor—There is no measurable accumulation of phosphate due to amino acids in the absence of hydroxylamine. It is worth

![Graph](http://www.jbc.org/)

**Fig. 3.** Orthophosphate, pyrophosphate, and hydroxamic acid formation as a function of Mg\(^{++}\):ATP ratio. 2 µmoles of each of twelve amino acids, 1.2 mmoles of NH\(_2\)OH, and 7 mg. of pH 5 enzyme in 1 ml. were incubated for 60 minutes. ATP was held constant at 10 µmoles and MgCl\(_2\) was varied as indicated. (Other experiments reveal that with Mg\(^{++}\):ATP below 0.1 the PP and hydroxamic acid are equal and decrease with decreasing Mg\(^{++}\):ATP.) These data are from one of three similar experiments.

restatement at this point that AMP inhibits neither the exchange nor the hydroxamic acid formation and, more important, that C\(^{14}\)-labeled AMP fails to exchange with ATP under conditions in which PP\(^{32}\)-ATP exchange is vigorous. Furthermore, as would be expected from equilibrium considerations, NH\(_2\)OH markedly inhibits PP\(^{32}\)-ATP exchange.

Although the above evidence indicates that the activated amino acid and AMP are bound to the enzyme in the absence of an acceptor, some attempt has been made to detect a trace of free AMP ~ amino acid compound. Five C\(^{14}\)-labeled amino acids (leucine, glycine, valine, isoleucine, phenylalanine) were incubated with ATP and the enzyme preparation. The products of the reaction, after deproteinization, were then adsorbed on
charcoal at pH 4.0. After six washings with buffer and inert amino acids, the charcoal was boiled for 60 minutes with 6 N HCl. These conditions would split the ribose from AMP and thus release any bound C¹⁴-amino acids into the supernatant solution. There was, however, no greater radioactivity released than in controls in which the enzyme was heated, Mg was omitted, or adenosine diphosphate replaced ATP.

**Separate Activation of Naturally Occurring L-Amino Acids**—Both PP-ATP exchange and hydroxamic acid formation are dependent upon L-amino acids. The D isomers are inactive and do not inhibit the activation of the L isomers. The extent of hydroxamic acid formation and exchange produced by a number of amino acids are reported in Table I. It can be seen

<table>
<thead>
<tr>
<th>Amino acid, 5 μmoles each</th>
<th>Per cent exchange</th>
<th>Hydroxamic acid formed μmoles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptophan</td>
<td>2.4</td>
<td>1.1</td>
</tr>
<tr>
<td>Leucine</td>
<td>14.7</td>
<td>1.0</td>
</tr>
<tr>
<td>Alanine</td>
<td>1.2</td>
<td>0.7</td>
</tr>
<tr>
<td>Lysine</td>
<td>0.2</td>
<td>0.3</td>
</tr>
<tr>
<td>Valine</td>
<td>5.1</td>
<td>0.1</td>
</tr>
<tr>
<td>All 5</td>
<td>17.3</td>
<td>3.0</td>
</tr>
</tbody>
</table>

Exchange was measured by incubating 4 mg. of pH 5 enzyme with 5 μmoles of ATP, 1 μmole of Mg++, and 4 μmoles of PP (containing 280,000 c.p.m.) in 1 ml. for 8 minutes at 37°. Hydroxamic acid formation was determined after incubating 9.7 mg. of protein with 10 μmoles of Mg ATP, 1.2 mmoles of NH₄OH, and the amino acids indicated in a volume of 1 ml. for 50 minutes at 37°.

that in both reactions the effect of individual amino acids is additive. Indeed, even at high concentrations, there is no evidence of competition between amino acids for activation sites. Two further points to be noted are (1) the great difference in activity for the various amino acids and (2) the fact that there is no parallelism between hydroxamic acid formation and exchange.

**Methionine-Activating Enzyme**—During earlier attempts to fractionate the soluble protein fraction with ammonium sulfate, a peak of activity for hydroxamic acid formation with the group of twelve amino acids was found between 35 and 45 per cent saturation. However, when the amino acids were tested individually, methionine accounted for essentially all of the activity. Table II shows that this ammonium sulfate fraction catalyzes the conversion of methionine to methionine hydroxamic acid with
concomitant production of approximately 2 orthophosphate equivalents. It should be noted that at the lower Mg\(^{++}\) concentration pyrophosphatase is inhibited and pyrophosphate accumulates as the product. This fraction

**Table II**

*Methionine Carboxyl Activation by Ammonium Sulfate Fraction from Rat Liver Soluble Protein Fraction*

<table>
<thead>
<tr>
<th>Products formed</th>
<th>Hydroxamic acid µmoles</th>
<th>P µmoles</th>
<th>PP µmole</th>
<th>F/2 + PP µmoles</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 µmoles Mg(^{++})</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No methionine</td>
<td>0.1</td>
<td>3.8</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>5 µmoles methionine</td>
<td>2.1</td>
<td>6.5</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>(\Delta)</td>
<td>2.0</td>
<td>2.7</td>
<td>0.2</td>
<td>1.6</td>
</tr>
<tr>
<td>4 µmoles Mg(^{++})</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No methionine</td>
<td>0.2</td>
<td>2.0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>5 µmoles methionine</td>
<td>1.7</td>
<td>3.4</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>(\Delta)</td>
<td>1.5</td>
<td>1.4</td>
<td>0.7</td>
<td>1.4</td>
</tr>
</tbody>
</table>

3.5 mg. of protein were incubated at 37\(^\circ\) for 50 minutes with 10 µmoles of ATP, 10 or 4 µmoles of Mg\(^{++}\), and 1.2 mmoles of NH\(_4\)OH. Methionine hydroxamic acid was used as standard.

**Table III**

*Nucleotide Specificity of Activation System*

<table>
<thead>
<tr>
<th>Nucleotide added</th>
<th>Hydroxamic acid formed µmole</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP 2.5 µmoles</td>
<td>0.79</td>
</tr>
<tr>
<td>CTP 2.0 &quot;</td>
<td>0.1</td>
</tr>
<tr>
<td>UTP 2.5 &quot;</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>ITP 2.5 &quot;</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>GTP 2.5 &quot;</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>None</td>
<td>0</td>
</tr>
</tbody>
</table>

4.6 mg. of the pH 5 enzyme were incubated at 37\(^\circ\) for 60 minutes with nucleotides, 1.2 mmoles of NH\(_4\)OH, and 24 µmoles of twelve amino acids.

also catalyzes a methionine-dependent PP\(^{32}\)-ATP exchange. The methionine hydroxamic acid has been identified by paper chromatography.

**Nucleotide Specificity**—Further progress in delineating the components of the amino acid incorporation system (9) has revealed that the washed pH 5 enzyme, when combined with crystalline ATP and microsomes that have been centrifuged out of a diluted 15,000 \(\times\) g supernatant solution,
no longer catalyzes incorporation of amino acids into protein. If the system is supplemented with GDP or GTP, however, activity is restored. The nucleotide is ineffective in the absence of ATP, and other nucleotides will not replace the guanosine derivatives. Thus it was of great interest to determine whether GDP or GTP is required for amino acid activation. The washed pH 5 enzyme was found to be fully active in the amino acid-dependent PP-ATP exchange with crystalline ATP and twelve L-amino acids, and GDP was found to have no effect on the exchange. In addition GTP cannot replace ATP as the source of energy for activation of the group of twelve amino acids tried (Table III).

Further Fractionation of pH 5 Enzyme—To determine whether separate enzymes mediate the activation of individual amino acids, several iso-
electrically precipitable fractions were tested for their activity toward a representative group of amino acids. Fig. 4 is typical of a number of such experiments. It can be seen that there is indeed a clear-cut difference in activity of the fractions toward the amino acids tested. There would appear to be separation of activity for leucine in the fractions obtained at pH 6.2 to 5.8. This would indicate that a separate protein mediates the activation of leucine. In another experiment, activity for alanine was also separated by isoelectric precipitation. With use of two of these

**Table IV**

Hydroxylamine Inhibition of Incorporation of L-Leucine-C\textsuperscript{14} into Protein

The figures are in counts per minute per mg. of washed protein determined as described previously (1).

<table>
<thead>
<tr>
<th>Hydroxylamine ((\mu)moles per ml.)</th>
<th>ATP precursors</th>
<th>Phosphoenolpyruvate, 10 (\mu)moles per ml.</th>
<th>Phosphocreatine, 20 (\mu)moles per ml.</th>
<th>Carbamyl phosphate* 10 (\mu)moles per ml. and kinase\textsuperscript{†}</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>79</td>
<td>88</td>
<td>87</td>
<td></td>
</tr>
<tr>
<td>0.05</td>
<td>70</td>
<td>88</td>
<td>87</td>
<td></td>
</tr>
<tr>
<td>0.2</td>
<td>48</td>
<td>35</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td>0.6</td>
<td>27</td>
<td>22</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>1.2</td>
<td>15</td>
<td>14</td>
<td>11</td>
<td></td>
</tr>
</tbody>
</table>

Each flask contained, in a final volume of 1.0 ml., 0.1 \(\mu\)mole of L-leucine-\(\text{C}^{14}\) \(82,000\) c.p.m. (supplied by Dr. R. B. Loftfield), 0.7 ml. of supernatant fluid (with 15.5 mg. of protein) centrifuged at 15,000 \(\times\) \(g\) from a rat liver homogenate prepared as described under "Experimental." The incubation time was 10 minutes at 37° under 5 per cent CO\textsubscript{2}-95 per cent N\textsubscript{2}.

* Kindly provided by Dr. L. B. Spector.
† A crude *Streptococcus faecalis* R extract, 0.2 mg. of protein per flask, supplied the necessary carbamate kinase. The extract was a gift from Dr. M. E. Jones.

fractions, one active for leucine and the other for alanine, it has been possible to demonstrate the appearance on paper chromatograms of FeCl\textsubscript{3}-reacting spots indistinguishable from known leucine and alanine hydroxamic acids respectively. Preliminary studies on heat inactivation of the pH 5 enzyme also point to separate activating enzymes.

**Hydroxylamine Inhibition of Incorporation of C\textsuperscript{14}-Amino Acid into Protein**—The fact that the pH 5 enzyme preparation which produces amino acid activation by forming enzyme-bound amino acyl \(\sim\) AMP complexes is also required for the incorporation of C\textsuperscript{14}-amino acids into protein in the cell-free system from liver suggests that this activation may be the first step in the incorporation reaction. Another suggestive piece of evidence is that the incorporation is inhibited by hydroxylamine (Table
IV) and that the inhibition roughly parallels the amino hydroxamic acid formation with increasing concentration of hydroxylamine. Table IV demonstrates that the inhibition is the same regardless of which of three different ATP generating systems is used.

**DISCUSSION**

The data presented in the foregoing support a hypothesis for amino acid activation which is schematically presented in Fig. 5. The R group of the amino acids and the adenine moiety of ATP would both be bound by the specific activating enzyme. This binding would create favorable conditions for a cleavage of ATP by an attack of the carboxyl oxygen upon the stable phosphate of ATP and ejection of the pyrophosphate. The bond energy would thus be retained in a carboxyl-phosphate linkage. The result would be an enzyme-bound, carboxyl-activated, amino acid ~ AMP compound. Magnesium may participate in the labilizing of the AMP ~ pyrophosphoryl linkage. Each activating enzyme or site appears to act independently, forming an AMP compound with a specific amino acid, without interference from other amino acids.

The enzyme-bound amino acid ~ AMP compound would then react with a natural cellular acceptor: either another nucleotide carrier or the nucleic acid of the microsome. The next step would be polypeptide
condensation, which appears to occur in the ribonucleoprotein particles of the microsome fraction (10). In the present experiments the artificial trap, hydroxylamine, permits the removal of the activated amino acid and regeneration of the enzyme for recycling. In any case, the point of cleavage of the carboxyl-phosphate linkage by the amine acceptor would leave the carboxyl oxygen on the AMP.

The PP-ATP exchange which depends upon amino acid, the failure of AMP to exchange with ATP, the lack of product accumulation in the absence of hydroxylamine, the appearance of amino hydroxamic acids and pyrophosphate in the presence of hydroxylamine, and the evidence suggesting separate activating enzymes are all consistent with this scheme.

A number of recent studies suggest that this mechanism of activation may be of quite general character when synthetic reactions involve a pyrophosphate split of ATP. Maas (11) has good evidence that the activation of pantoate for synthesis of pantothenate proceeds by an identical mechanism. Berg (12) has shown that the activation of acetate involves the initial formation of an acetyl \(\sim\) AMP compound. Evidence for the existence of an AMP \(\sim\) CO\(_2\) compound has been presented (13). Hilz (14) has observed the possible occurrence of an AMP \(\sim\) sulfate compound in a system in which ATP supplies the energy for sulfate activation. The ATP-dependent synthesis of benzoyl coenzyme A in hippuric acid synthesis seems to involve a pyrophosphate split of ATP (15). Finally, an amino acid-dependent PP-ATP exchange has been found in a variety of microorganisms (16). The present studies support the suggestion made by Lipmann 15 years ago (17) that the phosphate bond energy of ATP might be used for carboxyl activation of amino acids.

The extent of dissociation of the acyl \(\sim\) AMP compound from the specific enzyme seems to vary considerably in the different systems described above. It appears that, at least in the present system, dissociation of the AMP \(\sim\) amino acid compound from the enzyme is of such small extent that its free existence has thus far not been demonstrated. The interesting observation (Table I) that there is no parallelism between hydroxamic acid formed and PP-ATP exchange suggests that the amino acids differ not only in the rate at which they are activated (as measured by rate of exchange) but also in the extent to which the activation site is accessible to hydroxylamine (as measured by hydroxamic acid formation).

The stability properties and fractionation characteristics of this amino acid activation system closely parallel those of the soluble enzyme preparation required for incorporation of labeled amino acids into microsome protein. The inhibition of incorporation by hydroxylamine is also suggestive.

The rate of incorporation of leucine into whole liver protein in vivo has
been estimated as at least 5 \( \mu \)moles per gm. of protein per hour (3). In 1 gm. of liver protein there are about 400 mg. of soluble protein fraction which can form about 13 \( \mu \)moles of leucine hydroxamic acid per hour. This compares favorably with the figure for incorporation \textit{in vivo}.

The fact that the activation of the amino acids so far tested is specific for ATP and is uninfluenced by GTP suggests that the requirement for the latter nucleotide in the incorporation of amino acids into protein is at a stage following amino acid activation.

It is of interest that methionine can be activated at the carboxyl group by an ammonium sulfate-precipitable enzyme. Since this fraction is similar to one described by Cantoni (18) which contains the enzymes that synthesize adenosylmethionine from ATP and methionine, it is conceivable that carboxyl activation of methionine might be an intermediate stage in adenosylmethionine synthesis.

\section*{SUMMARY}

An enzyme preparation obtained from the soluble protein fraction of rat liver by precipitation at pH 5.2 to 5.1 has the following properties.

1. It catalyzes an exchange of PP\( ^{32} \) with ATP which is essentially L-amino acid-dependent. The exchange is dependent both on the concentration and the number of amino acids added.

2. When incubated with ATP, a mixture of L-amino acids, and a high concentration of hydroxylamine, hydroxamic acid is produced. The hydroxamic acids of leucine and alanine have been identified by paper chromatography. At the optimal Mg\( ^{++} \) concentration, 2 moles of orthophosphate are formed per mole of hydroxamic acid. At low Mg\( ^{++} \) concentrations, 1 mole of pyrophosphate is formed per mole of hydroxamic acid, and no orthophosphate appears. No products appear in the absence of hydroxylamine.

3. In all cases D-amino acids are inert.


5. Preliminary fractionation and heat inactivation studies suggest that separate enzymes are involved in the activation of several amino acids.

Incubation of a 35 to 45 per cent ammonium sulfate fraction of the soluble protein fraction of rat liver with methionine, ATP, and a high concentration of hydroxylamine leads to the formation of methionine hydroxamic acid. Other amino acids are not activated by this or other ammonium sulfate fractions.

The relation of these reactions to the activation of amino acids for protein synthesis is discussed.

The authors wish to thank Dr. Joseph C. Aub and Dr. Fritz Lipmann.
for advice and encouragement, and Miss Marion Horton for valuable technical assistance.

**BIBLIOGRAPHY**

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Paul C. Zamecnik


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