An enzymic mechanism for amino acid activation in animal tissues*

ZAMECNIK AND KELLER[1] have shown that rat liver soluble protein fraction is essential for the incorporation of ^{14}C-labeled amino acids into the protein of microsomes in the presence of ATP**. The data presented in the present paper suggest a mechanism for activation of amino acids in this supernatant fraction.

^{32}PP, when incubated with ATP, dialysed 100,000·g rat liver supernatant, $MgCl_2$ and KF becomes incorporated into ATP†, ††. This incorporation is enhanced two to three fold by the addition of a complete complement of pure L-amino acids. The incorporation into ATP of ^{32}P and ^{32}PP in microsomes and of ^{32}P in supernatant is unaffected by amino acids.

The enhancement of exchange is dependent both on the total concentration and the number of amino acids added. If the total concentration of amino acids is held constant, at 0.04 M, the PP-ATP exchange increases as the number of L-amino acids is increased (Table I). 0.4 ml of a rat liver 105,000·g supernatant fraction, prepared by the method of ZAMECNIK AND KELLER[1] with minor modifications, dialysed 16 hours against the buffered sucrose medium in which the homogenate was prepared, was incubated for 7 minutes at 37° in a volume of 1.1 ml with 5 μM ATP, 4 μM $MgCl_2$, 100 μM tris(hydroxymethyl)aminomethane buffer pH 7.6, 60 μM KF and 4 μM ^{32}PP, pH 7.5 containing 128,000 c.p.m. Every amino acid was chromatographically pure, they were added in groups of four, with a total concentration of 40 μM in each tube. (Changing the order of addition of the amino acids gave similar results.) The reaction was stopped with trichloroacetic acid and the ATP and PP separated and determined by charcoal adsorption according to the method of CRANE AND LIPMANN[2]. Under the above conditions, no ATP or PP was hydrolysed. % exchange = counts per minute per μM ATP divided by total counts per minute per μM ATP plus PP·100.

TABLE I

THE EFFECT OF INCREASING NUMBERS OF AMINO ACIDS ON PP-ATP EXCHANGE

Number of amino acids added	*Counts per minute per μM ATP*	*% exchange*
0	4038	28
4	5660	40
8	7960	56
12	8270	58
16	8970	63
20	9360	66

More than 2 μM/ml of each amino acid, on the average, does not further increase the exchange. Equivalent numbers and concentrations of D-amino acids are totally ineffective.

AMP is only slightly inhibitory to the system and ^{14}C-uniformly-labeled AMP fails to exchange with ATP***.

PP does not accumulate in the system in measurable quantity even though the PPase activity is completely inhibited by KF, and PP-ATP exchange is proceeding. However, in the absence of KF and in the presence of high concentration of *salt-free* NH_2OH, a mixture of L-amino acids produces a considerably increased "hydrolysis" of ATP and a corresponding appearance of two equivalents of P. Concomitantly there is produced a significant quantity of hydroxamic acid (Table II). Rat liver was homogenized with 1.5 volumes of 0.05 M KCl and the 100,000·g supernatant from this homogenate was dialysed for 20 hours against 100 times its volume of 0.05 M KCl. 1.0 ml of the supernatant was then incubated for 30 minutes at 37° C in a final volume of 2.0 ml with 10 μM MgK_2ATP, 100 μM tris buffer pH 7.6, and 2400 μM of freshly prepared salt-free hydroxylamine. An equimolar mixture of 12 amino acids (each of which alone failed to produce measurable quantities of hydroxamate — phe, val, leu, try, i-leu, lys, thr, ser, alan, his, arg, gly) was used at a total concentration of 12 μM/ml. Analyses:

(1) hydroxamate was measured directly on a 1.0 ml aliquot of the reaction mixture by the method of LIPMANN AND TUTTLE[3], using as an internal standard a mixture of the hydroxamates of six amino acids (leu, i-leu, val, lys, gly, ala)§.

(2) After killing the enzyme in the remaining 1.0 ml by boiling 2 minutes, P was determined by the method of FISKE AND SUBBAROW on a 0.1 ml aliquot and compared to a zero-time control.

(3) ATP was similarly determined on a 0.1 ml aliquot hydrolysed for 12 minutes in 1 N HCl. (In the absence of KF, all PP formed is split to P during the incubation.)

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** Abbreviations: ATP – adenosinetriphosphate; AMP – adenosinemonophosphate; PP – inorganic pyrophosphate; P – orthophosphate.

† The ^{32}PP was prepared by, and the gift of Dr. M. E. JONES.

†† Pabst K_2ATP was used routinely but similar results were obtained with the Sigma Chemical Company crystalline product.

*** Prepared from Schwarz ^{14}C-uniformly-labeled ADP enzymically (hexokinase and myokinase) and isolated by paper electrophoresis. The ^{14}C ADP was generously supplied by Dr. JOHN GERGELY.

§ American Cancer Society Scholar in Cancer Research.

TABLE II

ATP LOSS, AND HYDROXAMATE APPEARANCE IN THE PRESENCE OF AMINO ACIDS AND HYDROXYLAMINE (in μM/ml)

Addition	*Hydroxamate formed*	*ATP lost*	*Pi formed*
—	0	2.31	4.64
NH_2OH	0.34	1.39	2.78
AA	0	2.25	4.51
AA + NH_2OH	0.69	2.25	4.51
$\triangle$ due to AA alone	0	0	0
$\triangle$ due to AA in presence of NH_2OH	0.35	0.86	1.73

D-amino acids are inert here also. The appearance of hydroxamates, like the exchange, is dependent on the number of amino acids added, as well as the concentration. A purified enzyme from this supernatant, when incubated with leucine and NH_2OH, has yielded a product indistinguishable from leucine hydroxamate by paper chromatography.

These findings permit the following tentative formulation of the process by which amino acids are activated for protein synthesis. If E_1 is an activation site on an enzyme for a particular amino acid (AA_1), this site binds ATP in such a way as to labilize the AMP-pyrophosphoryl linkage:

(1) $E_1 \text{—} \overline{} + ATP \rightleftharpoons E_1 \text{—} \overline{AMP \text{—} PP}$

The amino acid then displaces the PP:

(2) $E_1 \text{—} \overline{AMP \text{—} PP} + AA_1 \rightleftharpoons E_1 \text{—} \overline{AMP \text{—} AA_1} + PP$

These two equilibria would permit PP-ATP exchange to occur only in the presence of amino acids, would account for the failure of AMP to inhibit the reaction or to exchange with ATP and the failure of PP to accumulate in the absence of an acceptor. The additive effect on exchange of different amino acids suggests a separate activation site for each.

In the presence of NH_2OH, the carboxyl-activated amino acid reacts to form the hydroxamate, while AMP and PP accumulate, since the enzyme is regenerated to cycle again:

(3) $E_1 \text{—} \overline{AMP \text{—} AA_1} + NH_2OH \rightarrow E_1 + AA_1 \text{—} NHOH + AMP$

(The possibility of the AMP$\sim$AA dissociating from the enzyme is not ruled out and is consistent with the results if the dissociation were of small extent.)

The above formulation is similar to that of MAAS[4] for the enzymic synthesis of pantothenic acid in a purified *E. coli* extract where detailed stoichiometry of the reaction has been worked out.

The natural intracellular counterpart of NH_2OH in reaction (3) above might be expected to be the amino group of amino acids or peptide chains in the microsomes where arrangement of amino acid sequence and condensation of peptide chains would occur[5]. Examination of the interrelationship of these two fractions is in progress in this laboratory.

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MAHLON B. HOAGLAND*

Medical Laboratories of the Collis P. Huntington Memorial Hospital of Harvard University, Massachusetts General Hospital, Boston, Mass. (U.S.A.)

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