PROPIONYL-CoA HOLOCARBOXYLASE SYNTHESIS FROM BIOTINYL ADENYLATE AND THE APOCARBOXYLASE IN THE PRESENCE OF AN ACTIVATING ENZYME

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The activation of propionyl-CoA apocarboxylase and the binding of C^{14}-biotin to proteins in cell-free extracts of biotin-deficient liver was reported by Kosow and Lane (1) to require ATP. We obtained similar results (2,3) and showed a requirement for two soluble enzyme fractions in holocarboxylase synthesis: (a) the apocarboxylase in extracts of biotin-deficient liver, and (b) an "apocarboxylase-activating enzyme" present in extracts of both normal and biotin-deficient tissues. The latter fraction, partially purified from pig liver, contained a "biotin-activating enzyme" similar to that reported in bacterial extracts which catalyzes a biotin-dependent P^{32}-pyrophosphate-ATP exchange and the conversion of biotin to its hydroxamate in the presence of ATP and hydroxylamine (4,5). Biotinyl adenylate, the intermediate apparently formed by the bacterial and liver biotin-activating enzymes, reacts with CoA to give biotinyl-CoA as a substrate for oxidation of the biotin side chain. The bacterial enzyme preparation does not substitute for the pig liver apocarboxylase-activating enzyme in holocarboxylase synthesis, and it could not be concluded at that time whether the biotin-activating enzyme present in the liver apocarboxylase-activating fraction played a role in this synthetic process (3). The requirement for two enzyme fractions has also been reported by Kosow et al. (6,7). These

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investigators concluded that a carboxyl-activated biotin was not involved in propionyl-CoA holocarboxylase synthesis and stated that synthetically prepared biotinyl adenylate failed to replace ATP and biotin in this synthesis.

We now wish to report that synthetically prepared biotinyl adenylate (B-AMP) of high purity is fully active in replacing ATP and biotin in holocarboxylase synthesis and that the apocarboxylase-activating enzyme is required in the reaction. Both enzyme fractions have been obtained largely free of an enzyme which catalyzes B-AMP hydrolysis.

Propionyl-CoA apocarboxylase was purified from acetone powder extracts of biotin-deficient rat liver as already described (6) except that chromatography on diethylaminoethyl(DEAE)-cellulose was carried out with gradient elution with 0.01 to 0.2 M potassium phosphate buffer, pH 7.4[1]. The apocarboxylase fraction was eluted with approximately 0.1 M buffer.

The apocarboxylase-activating enzyme was purified 70-fold from acetone powder extracts of normal rabbit liver. Extracts made with 10 volumes of 0.004 M Tris buffer, pH 7.35, were brought to 47% saturation with ammonium sulfate. The precipitate was dialyzed against 0.005 M phosphate, pH 7.4, and adsorbed on alumina C_γ (gel:protein ratio 0.14, weight basis) and eluted with 0.05 M phosphate buffer, pH 8.2. After dialysis the preparation was treated with calcium phosphate gel (gel:protein ratio 0.9) and the precipitate was discarded. The enzyme was then adsorbed on calcium phosphate gel (gel:protein ratio 3.2) and eluted with 0.03 M phosphate buffer, pH 8.2, containing 0.002 M biotin. DEAE-cellulose chromatography was then carried out with gradient elution, the enzyme being removed from the column with ca. 0.15 M phosphate buffer, pH 7.4. Purification of the apocarboxylase-activating enzyme was determined by assays for holocarboxylase synthesis in the presence of an excess of the apocarboxylase fraction. In all assays for holocarboxylase formation the apoenzyme fraction was preincubated for

[1]All buffers used in enzyme purification contained 0.01 M mercaptoethanol and 0.001 M ethylenediaminetetraacetate(EDTA).

10 minutes with avidin to inactivate endogenous holoenzyme. An excess of biotin was then added as well as ATP (or B-AMP) and activating enzyme to initiate holocarboxylase synthesis. Aliquots were subsequently assayed for propionyl-CoA-dependent $C^{14}O_2$ fixation by the procedure of Tietz and Ochoa (8). The results given represent the average of duplicate assays, corrected for control experiments in which ATP was omitted, and are expressed as mμmoles CO_2 fixed.

Biotinyl-5'-adenylate was prepared from AMP and d-biotin in 75% pyridine with dicyclohexylcarbodiimide by the general procedure of Berg (9). After precipitation with acetone, B-AMP was extracted with water at pH 5.5, concentrated *in vacuo* in a 40° bath to about 0.1 M, and passed at room temperature over a column of DEAE-cellulose previously washed with 0.1 M phosphate buffer, pH 7.4, and then with water. Stepwise elution with KCl was carried out, B-AMP appearing with 0.05 M KCl. This preparation contained acyl phosphate (determined by hydroxamate formation), adenine, vicinal hydroxyl groups (determined with periodate), and organic phosphate in the ratio 0.98 : 1.00 : 1.03 : 1.04 and exhibited a single hydroxamate-forming spot, R_f 0.64, on paper chromatography in isobutyric-water-ammonia (66:33:1).

The rates of holocarboxylase formation from apocarboxylase in the presence of B-AMP and ATP are shown in Fig. 1. The two compounds are equally effective up to about 30 minutes. The lowered effectiveness of B-AMP at longer intervals may be attributed to the presence of small amounts of B-AMP hydrolase in the preparations. The hydrolase, present in much higher amounts in less purified liver enzyme fractions, apparently requires no cofactors and is assayed by disappearance of the substrate as estimated by hydroxamate formation.

The requirements for the activity of B-AMP and ATP are shown in Table I. As would be expected from the data of Fig. 1, B-AMP is relatively more effective with shorter incubation periods. When half of the B-AMP is added at zero time and the other half at 1 hour, this compound is as active as ATP (Expt. 1). Omission of glutathione decreases holocarboxylase synthesis about 24% with each substrate. A complete dependency on glutathione was not shown since traces of

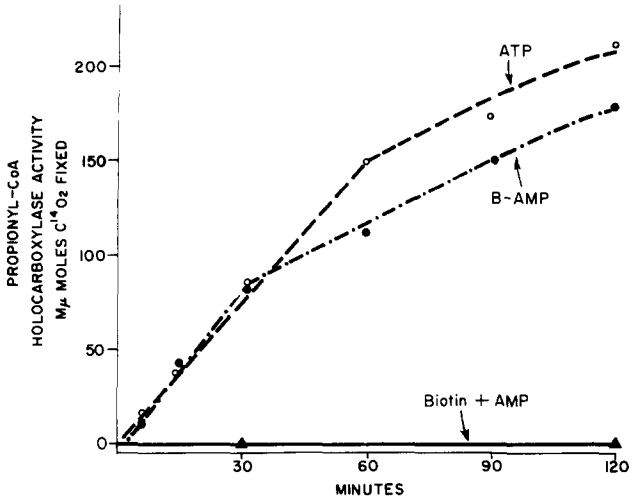

Fig. 1. Holocarboxylase formation as a function of time. Reaction mixtures containing (in μmoles) 50 Tris buffer (pH 7.7), 5 $MgCl_2$, 2.5 glutathione, 2 biotin, apoenzyme preparation (0.39 mg protein), activating enzyme preparation (0.35 mg protein), and 0.15 B-AMP or ATP, in a final volume of 1.0 ml, were incubated at 36° for the time indicated. Aliquots were assayed for propionyl-CoA carboxylase with $C^{14}O_2$ (8).

Table I

REQUIREMENTS FOR ACTIVITY OF BIOTINYL ADENYLATE AND ATP IN HOLOCARBOXYLASE SYNTHESIS

Expt. No.	System	Incubation (hours)	Propionyl-CoA dependent $C^{14}O_2$ fixation (mμmoles) with ATP	with B-AMP
1	Complete	2	237	224
	" (2 B-AMP additions)†		---	261
	No glutathione		180	169
2	Complete	3	162	98
	No Mg^{++}		107	65
3	Complete	1/2	90	88
	No apocarboxylase		3	0
	No activating enzyme		2	0

The complete reaction mixture contained in a typical experiment (in μmoles) 50 Tris buffer (pH 7.7), 5 $MgCl_2$, 2.5 glutathione, 2 biotin, apocarboxylase (0.25 mg protein), and activating enzyme (0.09 mg protein); ATP (2.0 in Expt. 1 and 2; 0.07 in Expt. 3) or B-AMP (0.17, 0.20, and 0.07 in Expt. 1 to 3, respectively) was added to give a final volume of 1.0 ml; EDTA (2.0) was added to those tubes having no added $MgCl_2$.

†One-half of the B-AMP was added at the beginning of the incubation and one-half at 1 hr.

mercaptoethanol were in the enzyme. Omission of $MgCl_2$ (Expt. 2) gives about 2/3 activity with each substrate. A complete metal dependency was not shown here or in earlier experiments, even in the presence of EDTA. With B-AMP, as with ATP, holocarboxylase formation is completely dependent on both enzyme fractions (Expt. 3). Biotin-activating enzyme purified over 100-fold from extracts of pig liver would not substitute for rabbit liver apocarboxylase-activating enzyme in the usual system containing apocarboxylase and ATP. Apparently the biotin-activating enzyme is not identical to the apocarboxylase-activating enzyme, or an additional protein removed during the purification of the former is required for the transfer of the intermediate to the apoenzyme.

Although it seemed unlikely that B-AMP is converted to ATP rather than functioning directly in holocarboxylase synthesis, the following experiments were carried out to examine this possibility: (a) Crystalline pyrophosphatase added to the complete system containing B-AMP does not decrease activity; thus ATP formation from B-AMP and traces of pyrophosphate seems unlikely. (b) The minimum concentrations of ATP and B-AMP adequate to support the maximum rate of holoenzyme synthesis in the usual system (containing some B-AMP hydrolase) are about 10^{-6} and 10^{-5} M, respectively. The addition of crystalline hexokinase and glucose to the assay system completely abolished the activity of 10^{-7} to 10^{-4} M ATP but did not significantly affect the activity of 10^{-4} M B-AMP. Suitable controls showed that the products of the hexokinase reaction, glucose-6-phosphate and ADP, were without effect. (c) Significant incorporation of radioactivity into the proteins was observed when biotin-C^{14} of high specific activity was employed with ATP, but not when biotin-C^{14} was added in the presence of non-radioactive B-AMP.

Our results at this time indicate that B-AMP may function in the final stage of holocarboxylase synthesis as follows:

$$\text{Propionyl-CoA apocarboxylase} + \text{B-AMP} \xrightarrow{\text{(apocarboxylase-activating enzyme)}} \text{propionyl-CoA holocarboxylase} + \text{AMP}$$

In a brief report Lynen and Rominger (10) have recently indicated that B-AMP is involved in acetyl-CoA apo-

carboxylase activation in a yeast enzyme fraction but that ATP and biotin exhibited no activity in the same preparation. On the contrary, the rabbit liver apocarboxylase-activating enzyme which we have purified about 70-fold is fully active with either ATP and biotin or with B-AMP in catalyzing holocarboxylase synthesis.

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