PROPIONYL HOLOCARBOXYLASE FORMATION: COVALENT BONDING OF BIOTIN TO APOCARBOXYLASE LYSYL ε-AMINO GROUPS*

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The participation of biotin as a prosthetic group of propionyl carboxylase is well-established (Halenz and Lane, 1960; Kaziro et al. 1960), however, the nature of its attachment to the holoenzyme has not yet been reported. The preparation of a cell-free enzyme extract (Kosow and Lane, 1961), which catalyzes the ATP-dependent formation of propionyl holocarboxylase from propionyl apocarboxylase and d-biotin, provided a useful system for studying the nature of the attachment of biotin to carboxylase. The earlier isolation of biocytin (ε-N-biotinyl-L-lysine) from yeast extract by Wright et al. (1952) provided a clue as to the nature of the bond involved. The present investigation indicates that d-biotin becomes covalently bonded to lysyl ε-amino groups of propionyl apocarboxylase during propionyl holocarboxylase formation.

Preparation of C^{14}-biotin-labeled Propionyl Carboxylase. Acetone powders prepared from biotin-deficient rat livers were extracted and ammonium sulfate fractionated as described by Kosow and Lane (1961). The ammonium sulfate-fractionated enzyme (550 mg of protein) was incubated with 800 μmoles of sodium phosphate buffer, pH 7.0; 60 μmoles

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of ATP and $MgCl_2$; 50 μmoles of GSH and 0.42 μmole of d-biotin-1-C^{14} (2.5×10^7 c.p.m. per μmole) in a total volume of 17 ml for 4 hours at 30°. Following incubation, the C^{14}-biotin-labeled carboxylase was precipitated by bringing the ammonium sulfate saturation to 60%. After centrifugation, the precipitate was redissolved and dialyzed overnight against 8 liters of 0.005 M phosphate, pH 7.0, containing 5×10^{-4} M GSH. The dialyzed enzyme was applied to a DEAE-cellulose column (2.5 x 20 cm) and the concentration of the eluting buffer raised from 0.005 M to 0.20 M phosphate, pH 7.0, by gradient elution . The eluate was collected fractionally, assayed for propionyl carboxylase (Halenz and Lane, 1960), and the fractions having peak carboxylase activity (approximately 60% of the total carboxylase activity eluted) were pooled. After precipitation at 60% ammonium sulfate saturation and centrifugation, the enzyme was redissolved in dilute phosphate buffer, pH 7.4.

Enzymatic Hydrolysis of C^{14}-biotin-labeled Carboxylase and Isolation of C^{14}-biocytin. The C^{14}-biotin-labeled propionyl carboxylase preparation (60 mg of protein; 8500 c.p.m.) was enzymatically hydrolyzed by incubation with 2 mg of Streptomyces griseus protease (Pronase, Calbiochem Corp; Nomoto et al., 1960); 120 μmoles of sodium phosphate, pH 7.4; 3 μmoles of carrier biocytin; and 0.15 ml of ethanol in a total volume of 3.0 ml for 48 hours at 37°. It had been previously determined that the protease neither hydrolyzed biocytin detectably nor catalyzed an exchange between d-biotin-1-C^{14} and biocytin in the presence or absence of human serum albumin under the conditions described above. The enzymatic hydrolysate was streaked on several sheets of Whatman 3 MM paper and chromatographed using the n-butanol-glacial acetic acid-H_2O solvent system (80:20:20, v/v). The band containing biocytin (R_f = 0.37) and also alanine and tyrosine was eluted and found to contain nearly 100% of the radioactivity of the C^{14}-biotin-labeled carboxylase before hydrolysis. Biotin has an R_f of 0.78 with this solvent system. In order to confirm the

identity of the C^{14}-labeled enzymatic hydrolysis product, part (2380 c.p.m.) of the concentrated R_f-0.37 eluate was chromatographed (ion-exchange chromatography) using a Beckman-Spinco amino acid analyzer according to the method of Spackman et al. (1958). The column effluent was collected fractionally and aliquots taken for ninhydrin analysis (Rosen, 1957) and for measurement of radioactivity by liquid scintillation counting. Fractions 1-34 contained 10 ml and 35-91 contained 3.4 ml. The elution pattern (see Figure 1) shows that biocytin is the only radioactive component eluted and constitutes over 70% of the radioactivity chromatographed. The identity of the 3 principal ninhydrin peaks ($A_{565\ m\mu}$) was established by chromatography of authentic alanine, tyrosine, and biocytin under identical conditions. Phenylalanine, which is normally eluted in the same region as biocytin, was completely removed prior to ion-exchange chromatography by preliminary paper chromatography (R_f = 0.7) as described earlier.

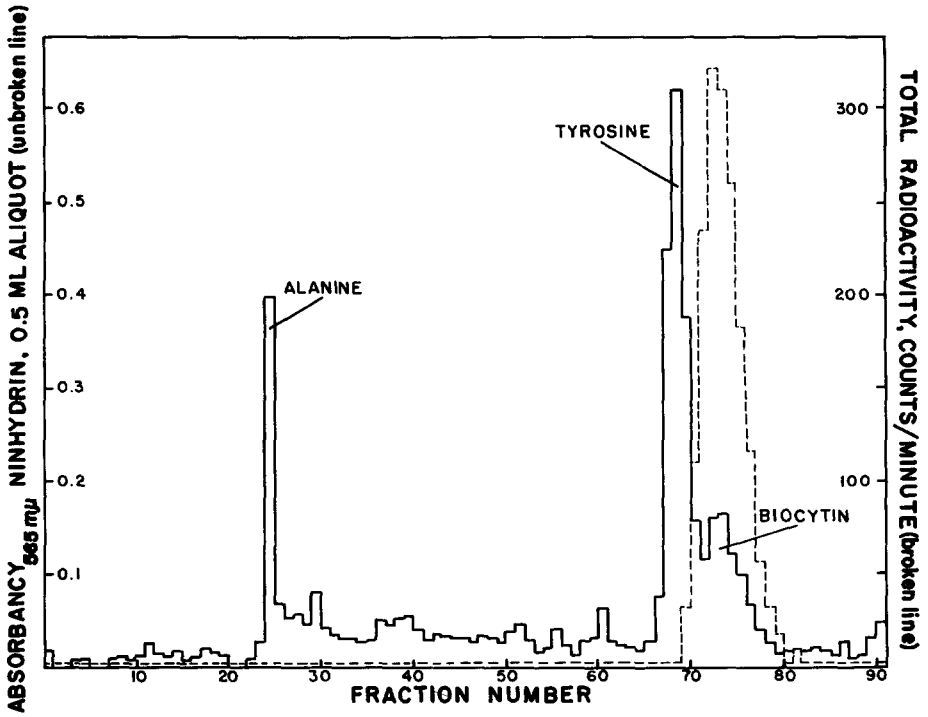

Figure 1. Elution Pattern of the Enzymatic Hydrolysis Product of C^{14}-biotin-labeled Propionyl Carboxylase

An experiment was conducted to determine whether biocytin is an intermediate in the formation of holocarboxylase from apocarboxylase and d-biotin. The data summarized in Table I, show that the enzyme system, which catalyzes holocarboxylase formation (Experiment 2) also catalyzes the ATP-dependent binding (Experiment I) of C^{14}-biotin to protein in the presence of L-lysine, but does not catalyze (Experiment I) the binding of C^{14}-lysine to protein in the presence of d-biotin. This would seem to rule out biocytin as an intermediate in holocarboxylase formation.

TABLE I

Comparison of ATP-dependent C^{14}-biotin and C^{14}-lysine Binding to Protein

Additions to basic reaction mixture[1]	C^{14}-biotin or C^{14}-lysine bound to protein	Propionyl carboxylase activity after incubatio
	c.p.m./tube[2]	units of carboxylase/tube
Experiment 1		
d-biotin-1-C^{14} + L-lysine	105	-
d-biotin-1-C^{14} + L-lysine + ATP (Mg^{++})	2115	-
L-lysine-U-C^{14} + d-biotin	51	-
L-lysine-U-C^{14} + d-biotin + ATP (Mg^{++})	89	-
Experiment 2		
d-biotin	-	0.058
d-biotin + ATP (Mg^{++})	-	0.600

[1] The basic reaction mixture consisted of: sodium phosphate buffer, pH 7.0, 40 µmoles; GSH, 1.2 µmoles; alumina C_γ gel supernatant enzyme, 2.5 mg of protein; and alumina C_γ gel eluate enzyme, 16 mg of protein. Other additions were ATP and $MgCl_2$, 1.6 µmoles; d-biotin-1-C^{14} (2.5×10^4 c.p.m. per mµmole) or d-biotin, 22 mµmoles (Experiment 1) and 80 mµmoles (Experiment 2); and L-lysine-U-C^{14} (2.5×10^4 c.p.m. per mµmole) and L-lysine, 22 mµmoles. Reaction mixtures in Experiment 2 were preincubated with 0.2 mg of avidin (2.5 units per mg) for 10 minutes at 30° to inactivate endogenous carboxylase after which excess d-biotin was added as indicated to initiate the reaction. Reaction mixtures (final volume 1.0 ml) were incubated for 4 hours at 30°. Following incubation the amount of C^{14}-biotin or C^{14}-lysine bound to protein (Experiment 1) was determined as described by Kosow and Lane (1961) or aliquots taken (Experiment 2) for carboxylase assays (Halenz and Lane, 1960).

[2] Average of duplicates.

The data presented indicate that the propionyl holocarboxylase-forming system catalyzes the covalent bonding of d-biotin to the lysyl ϵ-amino groups of the apocarboxylase.

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