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Some Aspects of Biotin Binding to Protein Catalyzed by Biotin-Deficient Chicken Liver Preparations[1] (37852)

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Cell-free preparations from biotin-deficient animals and microorganisms have been shown to catalyze an energy (ATP-)-dependent binding of ^{14}C-labeled D-biotin to protein (1–10). The enzymes catalyzing this reaction are termed holocarboxylase synthetases, which convert biotin-lacking apoproteins to active holocarboxylases in the presence of D-biotin and ATP. A number of holocarboxylase synthesizing systems have been investigated [see our review article (11)], and the following reaction mechanism has been proposed:

$$\text{Biotin} + \text{ATP} \rightleftharpoons \text{Biotinyl-5'-adenylate} + \text{PP}_i, \quad (1a)$$

$$\text{Biotinyl-5'-adenylate} + \text{apocarboxylase} \rightleftharpoons \text{holocarboxylase} + \text{AMP}. \quad (1b)$$

The biotinyl moiety is attached to an ϵ-amino group of a lysyl residue in the apoprotein molecule. Both the steps in the reaction are presumably catalyzed by one and the same enzyme, i.e., the synthetase.

If ^{14}C-labeled biotin is substituted for ^{12}C-biotin, it is obvious that holocarboxylases containing the radioactive biotin will be formed. In fact, biotin binding to apoproteins closely parallels the holocarboxylase synthesis (3, 8). Thus biotin incorporation is an indirect index of the extent to which the apocarboxylases are activated and hence also represents a measure of the activity of the synthetases responsible for the above process.

We have employed ^{14}C-biotin binding to protein as a simple and convenient means of investigating the distribution and some properties of the holocarboxylase synthesizing systems occurring in biotin-deficient chicken liver. This paper reports the results of these studies.

Materials and Methods. Animals. Biotin deficiency was produced in 1-day-old female chicks (Columbian female × New Hampshire male) by feeding a 20% spray-dried egg-white diet (12) *ad lib.* for a period of about 6 wk.

Chemicals. The various nucleoside phosphates and glutathione (GSH) were purchased from the Sigma Chemical Company. [Carbonyl-^{14}C]-D-biotin was obtained from Amersham/Searle Corp. Soluene TM 100 and enzyme-grade ammonium sulfate were products of Packard Instruments Co. and Mann Research Laboratories, respectively. Other chemicals used were of reagent grade.

Enzyme preparations. Mitochondrial and cytosolic fractions were obtained according to the following procedure. The 6-wk-old deficient animals were fasted overnight and killed by cervical dislocation. Livers were removed quickly and dropped into an ice-cold medium containing 0.3 *M* sucrose and 0.1 m*M* EDTA. Subsequent operations were carried out in the cold room (4°). The tissue, after being well minced, was homogenized for 3 min with 4 vol of the sucrose–EDTA medium (Waring blender). The resulting homogenate was strained through a layer of cheesecloth and centri-

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fuged initially at 800*g* for 15 min to remove nuclei and cell debris. Mitochondria were then sedimented at 13,000*g* for 30 min (Sorvall refrigerated centrifuge, model RC2-B) and supernatant was carefully decanted from the loosely clumped mitochondria at the bottom. The mitochondrial fraction was further sedimented at 25,000*g* for 30 min and subsequently washed twice with 2–3 vol of 0.1 m*M* EDTA. The pellet obtained after the second washing was suspended in a small amount of EDTA solution and lyophilized (Vortis Unitrap lyophilizer).

The 13,000*g* supernatant was recentrifuged at 100,000*g* for 60 min in a Beckman preparative ultracentrifuge, model L2-65. The clear supernatant obtained (cytosolic fraction) had a volume too large for direct lyophilization. Hence it was treated with solid ammonium sulfate to 70% saturation (all the enzyme activity was recovered by this procedure). The salt precipitate was dissolved in a minimal volume of 0.005 *M* phosphate buffer (pH 7.0), dialyzed against 100 vol of the same buffer overnight, and finally lyophilized as before.

Both the mitochondrial and cytosolic preparations were stored in jars containing anhydrous calcium chloride at −20°. Under these conditions there was no appreciable loss of activity over a period of several months. The mitochondrial preparation had some endogenous pyruvate and propionyl CoA carboxylase activities but no acetyl CoA carboxylase activity. The cytosolic preparation had only residual acetyl CoA carboxylase activity. The above findings indicate that the preparations were pure with respect to each other.

Assay of biotin binding activity. The procedure employed for measuring ^{14}C-D-biotin binding to protein was similar to the one described by Kosow, Huang and Lane (3). The reaction mixture in a total volume of 0.5 ml contained (μmoles): buffer of appropriate pH, 20; $MgCl_2$, 0.75; ATP, 0.75; GSH, 0.625; and ^{14}C-D-biotin (sp act, 23 μCi/μmole), 0.0055; and enzyme. The lyophilized preparations were extracted with 20 vol of 0.1 *M* Tris–HCl buffer (pH 7.5) for 60 min (30 min for cytosolic preparation) and centrifuged for 20 min at 25,000*g*. 60% saturated ammonium sulfate fractions of the extracts were used in the assay. Buffers used were Tris–HCl (pH 9.0) and Tris–maleate (pH 6.6) for determining mitochondrial and cytosolic activities, respectively. The reaction was started by adding the enzyme and stopped by chilling the samples in ice and then adding 1.5 ml of 5% ice-cold trichloroacetic acid. The precipitated protein was washed free of unreacted biotin essentially as described by

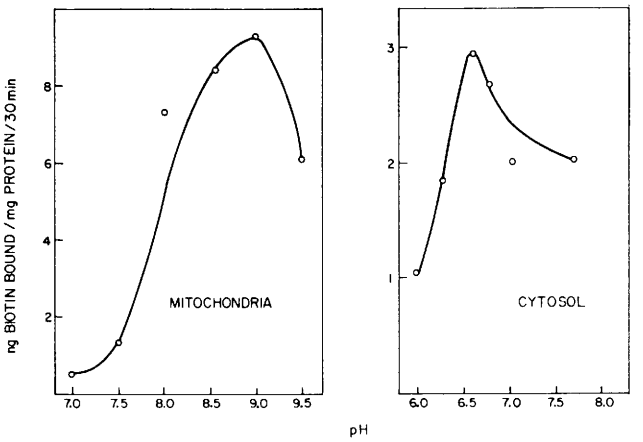

FIG. 1. pH profiles of mitochondrial and cytosolic biotin binding activities. Buffer systems used were: pH 6.0–7.0, Tris–maleate; and pH 7.5–9.5, Tris–HCl. Each point represents average of duplicate assays. Amount of protein put per tube: 1.43 mg (mitochondria) and 2.65 mg (cytosol).

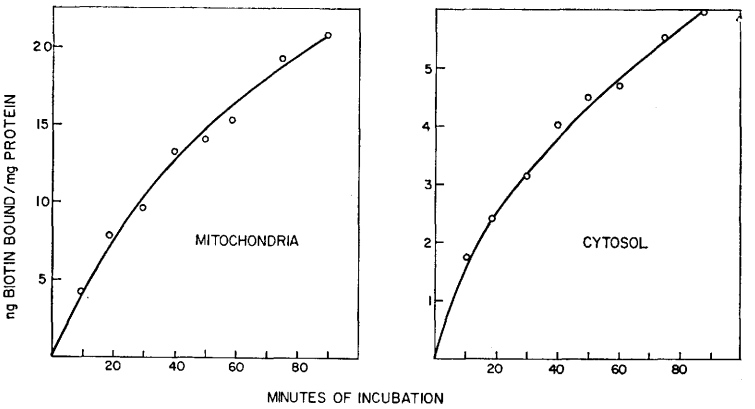

FIG. 2. Kinetics of biotin binding by the mitochondrial and cytosolic preparations. Each point represents average of duplicate assays. Amount of protein put per tube: 1.28 mg (mitochondria) and 1.85 mg (cytosol).

the procedure already referred to and dissolved in 0.5 ml of Soluene TM 100. Radioactivity was determined in a Packard liquid scintillation spectrophotometer using a toluene scintillant containing 0.5% 2,5-diphenyloxazole and 0.025% 1,4-bis-[2-(4-methyl-5-phenyloxazolyl)] benzene.

Protein was determined by the method of Lowry *et al.* (13). Enzyme activity is expressed in terms of nanograms of biotin bound per milligram of protein per 30 min (time of incubation used).

Results and Discussion. The pH profiles, kinetics, and effect of protein concentration on enzyme activities are shown in Figs. 1, 2, and 3, respectively. As shown in Fig. 1, the mitochondrial and cytosolic preparations exhibit different pH optima. The mitochondrial enzyme has peak activity around pH 9.0, whereas optimal activity for the cytosolic enzyme is observed at pH 6.6. Preliminary investigations had indicated that below pH 7.0 mitochondrial activity was quite low. The same was true for the cytosolic enzyme above 8.0. Specific activity of the cytosolic enzyme was low compared to the mitochondrial enzyme (2.94 and 9.32 ng of the biotin bound/mg of protein/30 min, respectively). This is understandable, since biotin deficiency does not cause as marked a reduction in acetyl CoA carboxylase activity as in the activities of pyruvate and propionyl CoA carboxylases (12). This implies that the level of acetyl CoA apocarboxylase (which is the one that binds ^{14}C-biotin) is very much lower than the levels of apocarboxylases present in mitochondria; hence the observed low cytosolic activity.

Requirements for the reaction. The effect of omission of the reaction components on

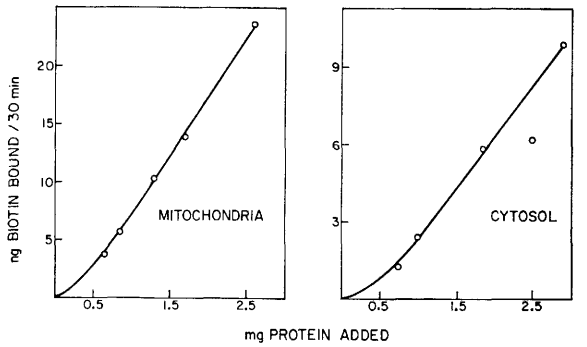

FIG. 3. Effect of protein concentration on the mitochondrial and cytosolic biotin binding activities. Each point represents average of duplicate assays.

TABLE I. Requirements for Biotin Binding.

Omission from the complete system	Biotin bound[a]			
	Mitochondrial enzyme[b]		Cytosolic enzyme[c]	
	ng/mg protein	% of control	ng/mg protein	% of control
None	8.98	100.0	3.91	100.0
ATP	0.90	10.0	0.73	18.7
$MgCl_2$	9.87	109.9	3.51	89.8
GSH	9.20	102.4	3.93	100.6

[a] Values given are averages of duplicates.
[b] 1.28 mg protein/tube.
[c] 2.03 mg protein/tube.

the extent of biotin incorporation is shown in Table I. Activities of both enzymes are reduced by more than 80% when ATP is excluded from the reaction mixture. The residual activity observed in the absence of ATP probably results from spontaneous (nonenzymatic) binding of ^{14}C-biotin to proteins. Such an energy-independent and nonspecific fixation of carboxyl-labeled biotin has been demonstrated to occur in chicken liver homogenates (2). Lack of $MgCl_2$ or GSH has negligible effect on biotin binding activity. Failure to observe any loss in enzyme activity in the absence of $MgCl_2$ could be explained on the basis that the enzyme preparations presumably contained enough Mg^{2+} ions to promote biotin binding.

Nucleoside triphosphate specificity. For a number of holocarboxylase synthesizing systems ATP seems to be an essential requirement (11). However, chicken liver pyruvate holocarboxylase (8) and propionyl CoA holocarboxylase (14) synthesizing systems can utilize other nucleoside triphosphates in place of ATP. This is true for the holoenzyme formation as well as for ^{14}C-biotin binding. Our results, given in Table II, are in conformity with the above findings. Interestingly, UTP is even more effective than ATP in stimulating biotin binding (21 and 42% higher activities for mitochondrial and cytosolic system, respectively). While GTP and ITP are more efficient with the mitochondrial enzyme than with the cytosolic enzyme, CTP exhibits the opposite effect. Possibility of ATP generation from the other nucleoside phosphates is not ruled out, but it does not explain the higher activities observed with UTP. Our observations on acetyl CoA holocarboxylase synthesis by chicken liver cytosol (to be reported elsewhere) also agree with these data. Thus a lack of a strict specificity towards ATP seems to be a general feature of chicken liver enzyme systems.

Effect of AMP and ADP. As shown in Fig. 4, AMP and ADP inhibit both mitochondrial and cytosolic biotin binding. At 2 m*M* concentrations (1 μmole/0.5 ml) the mitochondrial enzyme activity is reduced 44% by AMP and 29% by ADP. At the same concentration the corresponding inhibitions for the cytosolic enzyme are 34 and 38%, respectively. With increasing concentrations, both compounds produce progressive and quite marked decreases in enzyme activities. AMP is very much more inhibitory than ADP. Cazzulo *et al.* (15) have reported that AMP and ADP produced some inhibition of pyruvate holocarboxylase synthesis in a bacterial system.

Effect of citrate on cytosolic biotin binding. Citrate, which is well known to activate acetyl CoA carboxylase, exerts a slight stimulatory effect on biotin binding catalyzed by the cytosolic enzyme (Table III). However, significant stimulation is seen

TABLE II. Nucleoside Triphosphate Specificity of Biotin Binding Activity.

Nucleoside triphosphate used[a]	Biotin bound[b]			
	Mitochondrial enzyme[c]		Cytosolic enzyme[d]	
	ng/mg protein	% of control	ng/mg protein	% of control
ATP	8.00	100.0	3.18	100.0
CTP	2.25	28.1	1.21	38.0
GTP	4.23	52.9	0.68	21.5
ITP	3.64	45.5	0.53	10.4
UTP	9.65	120.6	4.49	141.5

[a] ATP was replaced by equimolar concentrations of the indicated triphosphates used.
[b] Values given are averages of duplicates.
[c] 1.28 mg protein/tube.
[d] 2.03 mg protein/tube.

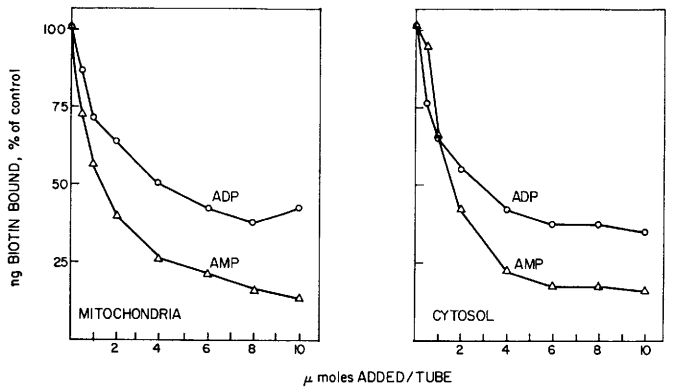

FIG. 4. Inhibition of mitochondrial and cytosolic biotin binding by AMP and ADP. Each point represents average of duplicate assays. 100% activity (control with no inhibitor added) is equal to 8.00 and 3.91 ng of biotin bound/mg of mitochondrial and cytosolic protein, respectively. Amount of protein put per tube: 1.28 mg (mitochondria) and 1.85 mg (cytosol).

only at citrate concentrations of 12 m*M* and above. Whether this is due to a direct effect of citrate on the synthetase or a secondary effect arising out of activation of the apocarboxylase itself is uncertain. In a like manner, acetyl CoA, which activates pyruvate carboxylase, is found to stimulate pyruvate holocarboxylase synthesis by an enzyme preparation from *B. stearothermophilus* (10, 16–18). But in this case preliminary evidence indicates that the activating effect is on the apocarboxylase rather than on the synthetase (18).

In the present study we have attempted to examine the intracellular distribution and some properties of the enzymes involved in ^{14}C-biotin binding to protein. As mentioned earlier, these enzymes are presumably the same as holocarboxylase synthetases. Of the three carboxylases known to be present in chicken liver, pyruvate and propionyl CoA carboxylases are located in the mitochondria, whereas acetyl CoA carboxylase is present in the cytosol. In the biotin-deficient animal all these would exist as inactive apocarboxylases. Upon administration of the vitamin, rapid conversion of the apoproteins to active holoenzymes takes place (12). The interesting question at this point is whether the various activation processes are brought about by a single synthetase or by different synthetases. Since the apocarboxylases are present in different cell compartments, it is reasonable to expect at least two synthetases to be operating. The one present in mitochondria would presumably catalyze both pyruvate and propionyl CoA holocarboxylase syntheses. The other, located in cytosol, would be responsible for formation of acetyl CoA holocarboxylase. Whether the mitochondrial apoproteins are activated by the same synthetase or different synthetases is also not known. Our data provide some evidence in support of the possibility of at least two synthetases being present. The differences in pH optima between the mitochondrial and cytosolic enzymes and their somewhat varied behavior towards nucleoside triphosphates suggest that the two synthetases may be different. However, purification and further investigation of the enzyme systems

TABLE III. Effect of Citrate on Cytosolic Biotin Binding.

Citrate added (μmoles)	Biotin bound[a]	
	ng/mg protein	% of control[b]
None	3.02	100.0
1.0	2.90	96.0
2.0	3.10	102.6
4.0	3.12	103.3
6.0	3.38	111.9
8.0	3.63	120.2
10.0	3.77	124.8

[a] Values given are averages of duplicates.

[b] Same as the reaction mixture described in the text. Each tube contains 2.03 mg protein.

is necessary to provide conclusive evidence.

Summary. Mitochondrial and cytosolic preparations obtained from biotin-deficient chicken liver catalyze incorporation of ^{14}C-labeled D-biotin to protein. The pH optima for the two enzyme systems are different. ATP is required for the reaction. Omission of GSH and $MgCl_2$ from the reaction mixture does not appreciably affect enzyme activity. Nucleoside triphosphates other than ATP are also effective in promoting biotin binding to protein. High concentrations of AMP as well as ADP inhibit the reaction. Citrate exerts a slight stimulatory effect on the activity of the cytosolic preparation.

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