Mutant Holocarboxylase Synthetase

EVIDENCE FOR THE ENZYME DEFECT IN EARLY INFANTILE BIOTIN-RESPONSIVE MULTIPLE CARBOXYLASE DEFICIENCY

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ABSTRACT Biotin-responsive multiple carboxylase deficiency is an inherited disorder of organic acid metabolism in man in which there are deficiencies of propionyl-coenzyme A (CoA), 3-methylcrotonyl-CoA, and pyruvate carboxylases that can be corrected with large doses of biotin. It has been proposed that the basic defect in patients with the early infantile form of the disease is in holocarboxylase synthetase, the enzyme that covalently attaches biotin to the inactive apocarboxylases to form active holocarboxylases. We have developed an assay for holocarboxylase synthetase in extracts of human fibroblasts using as substrate apopropionyl-CoA carboxylase partially purified from livers of biotin-deficient rats. Fibroblasts from the initial patient with the infantile form of biotin-responsive multiple carboxylase deficiency were shown to have abnormal holocarboxylase synthetase activity with a maximum velocity about 30–40% of normal, a K_m for ATP of 0.3 mM similar to the normal K_m of 0.2 mM, and a highly elevated K_m for biotin of 126 ng/ml, about 60 times the normal K_m of 2 ng/ml. These results show that the primary defect in this patient is a mutation affecting holocarboxylase synthetase activity, and thus a genetic defect of the metabolism of biotin.

INTRODUCTION

Biotin-responsive multiple carboxylase deficiency is an inherited disorder of organic acid metabolism (1–3) with a characteristic pattern of accumulation of metabolites caused by decreased activities of at least three different carboxylases: propionyl-coenzyme A (CoA) carboxylase (PCC),[1] 3-methylcrotonyl-CoA carboxylase (MCC), and pyruvate carboxylase (PC; 4). Patients have been classified into two broad groups on the basis of their clinical manifestations. The classic infantile form (1–2, 5–7) is characterized by an onset early in life, usually in the newborn period, of episodes of vomiting and ketoacidosis followed by coma and death in the untreated patient. A clinically distinct disorder (3, 8–10), often initially diagnosed as acrodermatitis enteropathica has a slightly more indolent onset, usually appearing 3–6 mo after birth, and is characterized by alopecia, localized dermatosis, keratoconjunctivitis, and ataxia. Subnormal levels of biotin have been found in the plasma and urine of juvenile patients suggesting an abnormality of biotin absorption or transport (10). In both forms of the disease large amounts of 3-hydroxy-isovaleric acid, 3-methylcrotonylglycine or 3-methyl-crotonic acid, 3-hydroxy-propionic acid, methylcitric acid, and lactic acid are found in body fluids as a result of deficiencies of MCC, PCC, and PC (1–3, 5–10). Both types respond dramatically to treatment with 10 mg/d or more or biotin (1, 3, 6–10).

Leukocytes from both types of patients display very low activities of the three carboxylases (PCC, MCC, and PC) that require biotin as a cofactor, and the activities increase on treatment with oral biotin (3, 7, 10). Cultured fibroblasts from the classic infantile form have low activities of the three carboxylases in vitro which can be increased with elevated concentrations of biotin in the culture medium (4). In contrast, fibroblasts from the juvenile form have normal activities of the carboxylases even at low concentrations of biotin in the culture medium (3, 8, 10). Since the attachment of biotin to all three enzymatically inactive apoenzymes to form active holoenzymes may be catalyzed by a single enzyme, holocarboxylase synthetase (HCS; 11), we investigated the possibility that the fundamental defect in biotin-responsive multiple carboxylase deficiency is in HCS. In this paper we present data that indicate

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[1] *Abbreviations used in this paper:* apo-PCC, apopropionyl-CoA carboxylase; HCS, holocarboxylase synthetase; holo-PCC, holopropionyl-CoA carboxylase; MCC, 3-methylcrotonyl-CoA carboxylase; PC, pyruvate carboxylase; PCC, propionyl-CoA carboxylase.

a defect in HCS in a patient with the early infantile disease.

METHODS

Studies were carried out on fibroblasts derived from the skin of J.R., the patient originally reported by Gompertz et al. (1), now a 9-yr-old boy. Fibroblasts were grown in Ham's F-10 medium (Irvine Scientific, Santa Ana, Calif.), supplemented with 10% fetal calf serum. The patient's carboxylases are present as active holocarboxylases in this medium, which contains 55 μg/liter of biotin. The normal fibroblast lines FS2 and FS3 were obtained from the foreskins of normal infants and grown in Eagle's minimal essential medium (Irvine Scientific) supplemented with 10% fetal calf serum. The 5 μg/liter of biotin in this medium gives fully active holocarboxylases for normal cells but not those of J.R. Propionyl-CoA was prepared by the method of Simon and Shemin (12). Affinity columns of avidin coupled to Sepharose 4B were prepared by the method of Landman and Landman (13) with the exception that avidin concentration was increased to 5 mg/ml, and could bind about 10 μg biotin/ml of gel. All other reagents were purchased from Sigma Chemical Co., St. Louis, Mo., except Na $H^{14}CO_3$ which was from New England Nuclear, Boston, Mass.

Fibroblast extract preparation. Cells were harvested by trypsinization, washed twice with 50 mM Tris HCl buffer pH 7.4 containing 0.9% NaCl by centrifugation and diluted to 12.5×10^6 cells/ml with 50 mM Tris HCl buffer pH 8.0, 3 mM EDTA, and 2.5 mM reduced glutathione (GSH). The cells were lysed by sonicating for 2 × 10 s at 30 W using a Sonifier Cell Disruptor (Heat Systems-Ultrasonics, Inc., Plainview, N. Y.). Approximately 0.5–1.0 ml of fibroblast extract was passed through an 0.5 × 5-cm avidin-Sepharose column equilibrated with 200 mM Tris HCl buffer, pH 8.0, 16 mM $MgCl_2$, 100 mM KCl, 0.2 mM Na_2 EDTA. HCS eluted from this column within the first 2 ml of buffer, whereas holocarboxylases and biotin were retained. Negligible amounts of apopropionyl-CoA carboxylase (apo-PCC) were found in the extracts of both patient and normal fibroblasts. In general experimental values for radioactivity representing holopropionyl-CoA carboxylase (holo-PCC) activity were >10 times background, while the counts representing possible residual apo-PCC in these fractions were only 10% over background. The error of the experiment is greater than that. Preparations were used immediately but were stable for several days at 4°C.

Apo-PCC preparation. Weanling male Sprague-Dawley rats (Hilltop Lab Animals, Inc., Chatsworth, Calif.) were maintained on a biotin-free diet (ICN Nutritional Biochemicals, Cleveland, Ohio) for at least 3 wk, at which time the activity of holo-PCC in liver was <10% of normal. Two rats were killed by decapitation and their livers homogenized in 3 vol of ice-cold buffer P (20 mM potassium phosphate, pH 7.0, 1 mM EDTA, 1 mM GSH), to which sucrose was added to a final concentration of 2.25 M. The liver homogenate was centrifuged at 600 g for 10 min to remove cellular debris. Its supernatant was centrifuged at 10,000 g for 15 min and the resulting mitochondria-containing pellet was washed in an equal volume of buffer P containing 0.25 M sucrose by centrifugation. The washed pellet was suspended in 20–30 ml of buffer P without sucrose and sonicated twice for 20 s each at 40 W. The sonicate was centrifuged at 106,000 g for 1 h in a Beckman L3-50 ultracentrifuge (Beckman Instruments, Inc., Fullerton, Calif.). The specific activity of apo-PCC in the homogenate and sonicated pellet was determined by measuring holo-PCC activity after preincubation with and without excess biotin in the presence of ATP. The holo-PCC activity in the presence of biotin minus the holo-PCC in the absence of biotin represented the apo-PCC that was converted to holo-PCC by the HCS in these preparations. The resulting supernatant fluid was applied to a DEAE-Sepharose 6B column (2.5 × 9 cm) previously equilibrated with buffer P and eluted under pressure (Cole Scientific Pump, Calabasas, Calif.) at 2 ml/min using a linear gradient of 300 ml buffer P and 250 ml 0.3 M KCl in buffer P.

Fractions were collected and their HCS, apo-PCC, and holo-PCC activities determined. Aliquots were assayed by the procedure described below for holo-PCC activity after preincubation with and without biotin and ATP. The activity without biotin represented holo-PCC. The activity with biotin minus the activity without biotin represented the amount of apo-PCC converted to holo-PCC, an indirect measure of the HCS which coeluted.

To assay for apo-PCC, a cytosolic preparation of rat HCS was made. Livers were obtained from rats fed a normal diet and thus having a minimum of apo-PCC. The livers were homogenized in buffer P containing 0.25 M sucrose and centrifuged at 13,000 g for 20 min to remove the mitochondria that contained holo-PCC. The supernatant was centrifuged at 100,000 g for 1 h, concentrated to 1 ml, and passed through an avidin affinity column to remove any remaining holo-PCC. The eluate from the column was used as a source of rat HCS, and was incubated with fractions from the DEAE-Sepharose column, biotin, and ATP to detect apo-PCC.

It was found that apo-PCC coeluted with holo-PCC between 220 and 350 ml; so the fractions containing apo-PCC were detected by assaying holo-PCC. Fractions enriched in holo-PCC activity were pooled and concentrated by precipitation with ammonium sulfate at a final concentration of 55% saturation.

The precipitate was redissolved in about 3 ml of buffer P, applied to a column of Sepharose 6B (1.5 × 75 cm), and eluted with additional buffer P. The lower molecular weight rat HCS was separated from the higher molecular weight apo-PCC and holo-PCC on this column. Fractions from the Sepharose 6B columns were assayed for holo-PCC activity after preincubation with and without biotin and ATP. The activities of holo-PCC were not increased by the incubation with biotin, indicating separation of apo-PCC and HCS. To verify that apo-PCC coeluted with holo-PCC, the fractions were incubated with the preparation of rat cytosolic HCS and ATP, with and without biotin. The holo-PCC activity increased with biotin demonstrating the coelution of apo-PCC with holo-PCC. The elution volume of HCS was later determined by incubating a preparation of apo-PCC with biotin, ATP, and aliquots from the Sepharose 6B fractions. Fractions containing holo-PCC activity, which eluted between 60–70 ml, were pooled and passed through an avidin-Sepharose column (0.5 × 15 cm), equilibrated, and eluted with buffer P. Apo-PCC in the eluate was assayed by preincubation with the rat cytosolic HCS with and without biotin and ATP. The difference represented apo-PCC. Holo-PCC was retained on the column while apo-PCC was eluted within the first 10 ml of buffer P. Apo-PCC was stored in 3.2 M ammonium sulfate and was stable for about 3 wk at 4°C, but was much less stable when frozen. A comparison of apo-PCC concentration assayed with excess rat cytosolic HCS, ATP, and biotin showed that apo-PCC was purified ~200-fold through this entire procedure. The final preparation contained negligible amounts of holo-PCC or other holocarboxylases. Apo-MCC was absent but apo-PC was present in some preparations in concentrations as high as 10% of the apo-PCC.

HCS assay. To assay for the activity of human HCS, fibroblast preparations were combined with rat apo-PCC, biotin, and ATP to generate active holo-PCC which was then

measured by a modification of the method of Tietz and Ochoa (14). Earlier studies have shown an absence of species specificity for the HCS reaction with apocarboxylase (11). Holo-PCC was formed by mixing 30 μl of the fibroblast HCS preparation (4×10^5 cells, 0.075 mg protein) with an excess of apo-PCC and varying amounts of biotin in buffer T (final concentrations 100 mM Tris HCl, pH 8.0, 8 mM $MgCl_2$, 50 mM KCl, 0.1 mM Na_2 EDTA, 2.5 mM GSH, and 3 mM ATP) in a final volume of 300 μl. This was incubated 60 min at 30°C, and the reaction stopped on ice. The holo-PCC formed was assayed by adding 150 μl buffer T, propionyl-CoA (0.77 mM final concentration), $NaH^{-14}CO_3$ (3.3 mM final concentration, specific activity 7.0 mCi/mmol), and water to make a final volume of 600 μl. The reaction mixture was incubated 20 min at 30°C and stopped on ice, followed by the addition of 60 μl 20% trichloroacetic acid in a hood to remove unreacted $NaH^{14}CO_3$ ad $^{14}CO_2$. The solutions were transferred into scintillation vials and dried under a heat lamp for 6 h. The residues were dissolved in 1 ml water and 10 ml Bray's solution (15), and the acid nonvolatile radioactivity ([^{14}C]methylmalonyl-CoA) measured in a Beckman LS-250 liquid scintillation counter (Beckman Instruments). Liquid partition chromatography (16) showed that at least 90% of the acid nonvolatile radioactivity measured was the desired product [^{14}C]methylmalonyl. Blanks were assayed identically except that biotin was omitted. HCS activities were expressed as holo-PCC activity (picomoles $H^{14}CO_3$ fixed per minute per milligram protein). Proteins were determined by the procedure of Lowry et al. (17). Michaelis kinetic constants were calculated by fitting the data to the hyperbolic Michaelis-Menten curve with bilinear regression (18, 19).

RESULTS

The assay for normal human fibroblast HCS was found to be linear with time for up to 90 min of incubation at 30°C, and a standard incubation time of 60 min was chosen. Fig. 1 shows the pH optimum for fibroblast HCS with the highest activities at pH 8.0–8.5. The concentration of Tris HCl buffer at the different pH values was 22 mM during the incubation of the fibroblast extracts with apo-PCC, biotin, and ATP, and the pH values were adjusted to pH 8.0 with the 200 mM Tris HCl buffer for the assay of the product holo-PCC activity. HCS activity is highly dependent on buffer concentration. Decreasing the Tris-HCl concentration to 22 mM causes a large decrease in activity. Therefore, the specific activities obtained in the determination of the pH optimum were lower than those in Figs. 2 and 3 and Table I. Since pH 8.0 is near the optimum for both HCS and holo-PCC, this pH was chosen for the standard assays of HCS. Activity was found to increase linearly with increasing amounts of apo-PCC under conditions in which apo-PCC was not present in saturating amounts. The amount of apo-PCC required for saturation was determined experimentally for each preparation and ranged from 0.07 to 0.30 mg of protein. Activity was linear with the amount of fibroblast extracts from 0.01 to 0.07 mg of protein (1×10^4 to 1×10^6 cells) for both the controls and the patient (Fig. 2).

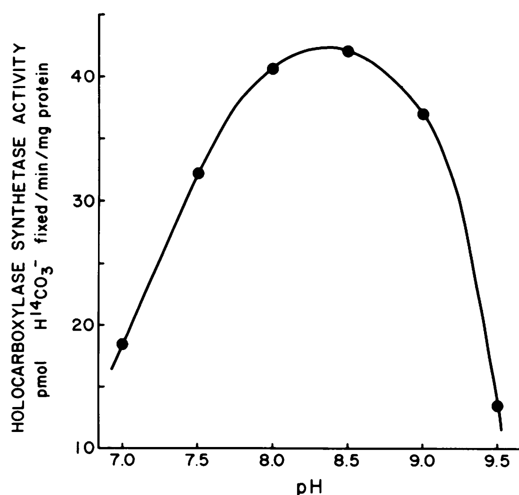


FIGURE 1 pH optimum for normal human fibroblast HCS. The assays were performed as described in Methods except for the incubation of 0.052 mg of protein of fibroblast extract with apo-PCC, 1,000 ng/ml of biotin, and 3.0 mM ATP in 20 mM Tris HCl at the different pH values. The assay for the product holo-PCC was at pH 8.0 in 100 mM Tris HCl.

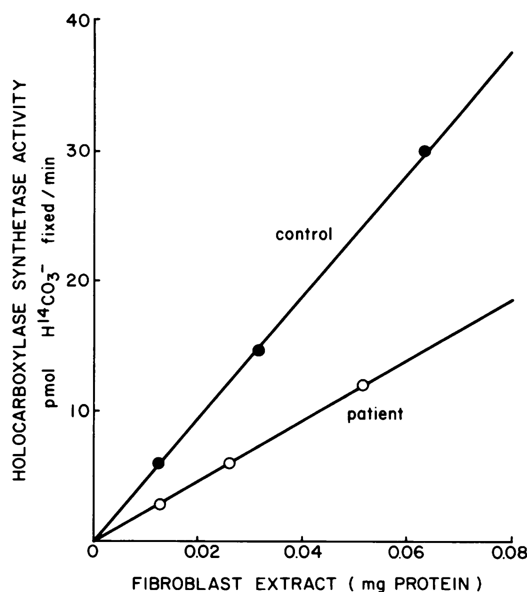


FIGURE 2 Linearity of HCS activity with amount of human fibroblast extract. Assays were performed as described in Methods with 1,000 ng/ml of biotin and 3.0 mM ATP.

The mean activity of HCS in fibroblast extracts of the patient was 30–40% of the mean normal activity at saturating levels of substrates (Table I). In addition to the low V_{max}, the HCS of the patient had a much higher K_m for biotin (126 ng/ml) than the control mean of 2 ng/ml (Fig. 3, Table I). The mean value for the patient was 60 times that of the controls. The K_m for ATP was similar for the patient and controls at 0.2–0.3 mm.

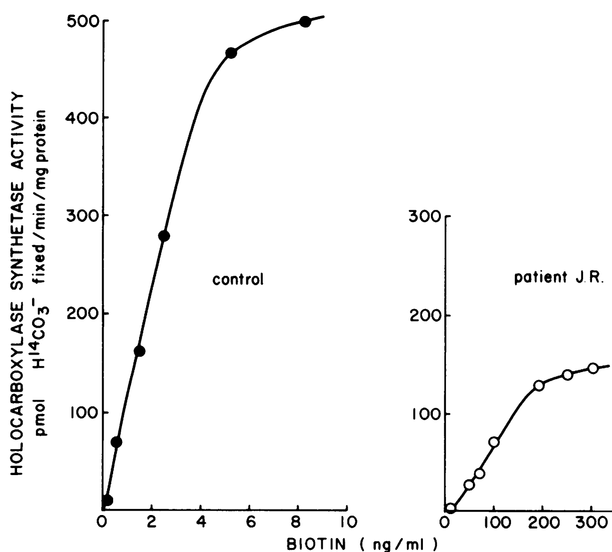
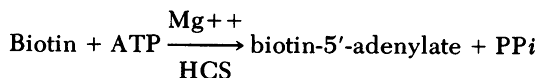
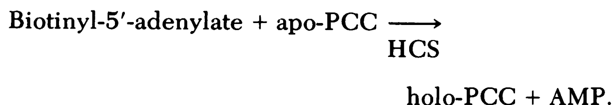


FIGURE 3 Activity of human fibroblast HCS as a function of the concentration of biotin. The assays were performed as described in Methods with 0.082 mg of protein for the normal and 0.046 mg of protein for the patient.

DISCUSSION

HCS covalently attaches biotin to a number of apocarboxylases to form enzymatically active holocarboxylases (11, 19). The reaction for PC is as follows:





Following activation by ATP, biotin forms an amide bond with the ϵ-amino group of a lysine residue on the apo-PCC to form the holo-PCC.

Data obtained with rat apo-PCC as the substrate for human fibroblast HCS from controls and the patient with biotin-responsive multiple carboxylase deficiency provide direct evidence of a genetic defect in holocarboxylase activity. The data correlate well with other clinical and laboratory information. The patient had an excellent clinical response to biotin (1) and has remained well while receiving biotin. However, it remains possible to detect abnormal metabolites in his urine resulting from low PCC and MCC activity even during biotin treatment (2). This observation may be consistent with the maximum activity for HCS in J.R. of 30–40% of normal and the very high K_m for biotin. The similar affinities for HCS for ATP in the patient and controls suggest that the major defect in the mutant HCS is in the binding site for biotin.

The K_m for biotin of 2 ng/ml (8 nM) obtained for HCS in the normal fibroblast cell lines was similar to the range of 0.5–3 ng/ml (2–12 nM) that we have found with similar assays for HCS in freshly isolated lymphocytes from five controls. It was also similar to the K_m for biotin of 1 nM reported for HCS from rabbit liver (20). It is of interest to note that the concentration of biotin in normal human plasma, ~0.2–0.8 ng/ml (or 0.8–3.0 nM; 21), are somewhat lower than the K_m of the normal human HCS. It may be that normal levels of biotin are not sufficient for optimum HCS activity in vivo or that intracellular concentrations of biotin are higher than in plasma. The elevated K_m of 126 ng/ml for biotin in the patient would be consistent with essentially no activity of the enzyme in vivo under conditions of the normal concentrations of biotin. On the other hand, treatment with 10 mg/d of biotin is capable of raising the concentration of biotin in plasma well above the abnormal K_m of the patient (10). The normal K_m for biotin of 2 ng/ml is consistent with the activity of the holocarboxylases in normal fibroblasts being independent of the biotin concentration from 5 ng/ml (minimal essential medium + 10% fetal calf serum)

TABLE I
Holocarboxylase Synthetase Kinetics in Extracts of Fibroblasts from Normal Controls and the Patient with Biotin-responsive Multiple Carboxylase Deficiency

	Variable substrate			
	Biotin		ATP	
	V_m*	Biotin K_m (ng/ml)	V_m*	ATP K_m (mM)
Controls				
FS-2	572	0.81	528	0.18
	523	3.20	418	0.19
	539	1.44		
Mean	545±25	1.82±1.24	473±78	0.19±0.01
FS-3	343	0.95		
	350	2.52		
	315	3.53		
Mean	336±19	2.33±1.30		
Mean control	440	2.08	473	0.19
Patient				
J.R.	248	79	152	0.50
	191	109	104	0.11
	109	191		
Mean	183±70	126±58	128±24	0.31±0.28

* Holocarboxylase synthetase velocities expressed as holo-PCC activity in picomoles $H^{14}CO_3^-$ fixed per minute per milligram protein. Mean values±1 SD.

to 55 ng/ml (F-10 + 10% fetal calf serum) in the culture media. The elevated K_m for biotin of 126 ng/ml for J.R. is consistent with the activities of the holocarboxylases being essentially zero at 5 ng/ml of biotin in the medium (1/25 of the K_m) but normal at 55 ng/ml of biotin in the medium (½ of the K_m).

The parallel response of the activities of the three mitochondrial carboxylases, PCC, MCC, and PC in the fibroblasts of the patient cultured in media with different concentrations of biotin (4) together with the evidence for an abnormal holocarboxylase with apo-PCC as substrate, implies that a single HCS attaches biotin to all three apocarboxylases. It is not known whether the cytoplasmic enzyme apo-acetyl-CoA carboxylase is activated by the same HCS.

Genetic complementation analysis with fused fibroblasts (4) has shown that the same genetic defect is present in the patient J.R. and another patient with the infantile form of biotin-responsive multiple carboxylase deficiency (5). The demonstration of a mutant HCS in J.R. therefore confirms the defect in HCS previously proposed without proof for this family (6). Preliminary assays indicate a normal HCS in fibroblasts of a patient with the juvenile form of biotin-responsive multiple carboxylase deficiency (10). This is consistent with normal carboxylase activities in the fibroblasts cultured with low concentrations of biotin. These results suggest that the primary defect of the metabolism of biotin in the juvenile form of biotin-responsive multiple carboxylase deficiency is not in the activity of HCS but may be in biotin absorption or transport. The primary defect of biotin metabolism in the infantile form of biotin-responsive multiple carboxylase deficiency is clearly an abnormal HCS and an appropriate name for this form of the disorder might be HCS deficiency.

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