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Evidence for a Defect of Holocarboxylase Synthetase Activity in Cultured Lymphoblasts from a Patient with Biotin-Responsive Multiple Carboxylase Deficiency

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SUMMARY

We report here the expression of biotin-responsive multiple carboxylase deficiency in cultured lymphoblasts of a patient whose fibroblasts belong to the *bio* genetic complementation group. Cultured lymphoblasts from the patient lost propionyl-CoA carboxylase (PCC) and β-methylcrotonyl-CoA carboxylase (MCC) activities at a faster rate than normal cells when grown in biotin-deficient medium. Recovery of normal PCC and MCC activities, which was independent of protein synthesis, required a 2,500-fold higher biotin concentration than that required by normal lymphoblasts. Holocarboxylase synthetase activity was detected in cell-free extracts through the biotinylation of endogenous apo-PCC in the presence of ATP to form active holo-PCC. While the apo-PCC in extracts of normal biotin-starved lymphoblasts could be activated to 28% of maximal activity, extracts of patient lymphoblasts did not exhibit any ATP and biotin-dependent increase in PCC activity. A normal cell extract, cleared of apocarboxylases by immunoprecipitation, stimulated the PCC activity of a patient cell extract 20-fold. These results indicate that the apoenzyme in *bio* cells is normal and that the defect lies in the holocarboxylase synthetase.

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INTRODUCTION

Biotin-responsive multiple carboxylase deficiency is a metabolic disorder in which patients present in infancy with alopecia, dermatitis, ketoacidosis, and the excretion of abnormal organic acids. Both the clinical and biochemical anomalies are dramatically resolved with pharmacological doses of biotin [1–9]. All the abnormal metabolites can be linked to the deficiency of three mitochondrial biotin-dependent carboxylase activities: propionyl-CoA carboxylase (PCC, E.C.6.4.1.3.), β-methylcrotonyl-CoA carboxylase (MCC, E.C.6.4.1.4.), and pyruvate carboxylase (PC, E.C.6.4.1.1) [10]. All three carboxylases have in common the presence of a covalently bound biotin prosthetic group. Conversion of the inactive apocarboxylase to the active holocarboxylase by the addition of biotin is mediated by a holocarboxylase synthetase [11].

In previous work, we examined the activities of PCC, MCC, and PC in cultured fibroblasts from two biotin-responsive patients [10]. All three enzyme activities were unmeasurable when the cells were cultured in a biotin-deficient medium in which normal fibroblasts retained full activity. All three activities were recovered after transfer of cells to biotin-rich medium. Complementation analysis established the existence of the complementation group *bio* [10], clearly distinct from groups comprised of patients missing only PCC [12] or PC [13] activity. Here we provide evidence that an individual who was assigned to the *bio* complementation group has a defect of the holocarboxylase synthetase. We have shown that the multiple carboxylase defect is expressed in lymphoblast cultures from the patient, that it can be corrected by growth in biotin-rich medium, and that the apocarboxylases in the patient cells appear to be normal and accumulate under biotin-restricted conditions. Further, we have developed a method to detect synthetase-dependent apo-PCC activation in lymphoblast extracts. We compared the activation capacity in extracts of normal and patient lymphoblasts and report here that holocarboxylase synthetase activity is deficient in the patient cells.

MATERIALS AND METHODS

Source and Growth of Cells

Patient J. T. is a biotin-responsive patient diagnosed and treated at The Hospital for Sick Children in Toronto. She presented at the age of 7 weeks with dermatitis, hypotonia, alopecia, and developmental delay. Her urinary organic acid profile showed major peaks of lactate, 3-hydroxyisovalerate, β-methylcrotonate, and methylcitrate. She was treated with biotin orally (10 mg/day) and improved dramatically. The lactic acidosis, skin rash, and abnormal organic acids disappeared within 10 days, and at age 1 year (still on biotin therapy), somatic and psychomotor development was normal for her chronological age. Patient fibroblasts showed a complete deficiency of PCC, MCC, and PC activities when grown under biotin-restricted conditions, but normal levels of each enzyme activity when grown in biotin-rich medium. Her cells were assigned to the *bio* complementation group using heterokaryon analysis [10].

Permanent lymphoblast cultures were started from a normal individual and patient J. T. by Epstein-Barr virus transformation according to the method of Glade and Broder [14]. Lymphoblasts were cultured in 200-ml spinner flasks and were fed twice per week with either α medium (α-MEM [15], Gibco, Grand Island, N.Y., containing 0.1 mg/l biotin and 15% fetal calf serum [FCS]) or biotin-restricted medium (α-MEM lacking biotin, 15% FCS,

and 30 U fresh avidin [Sigma, St. Louis, Mo.]). The latter medium contains about 1.8–2.8 μg/l biotin [16] that is sequestered by the added avidin [17]. There was no change in cell growth rate or viability when cells were cultured in the biotin-restricted medium. One hundred U penicillin and 100 μg streptomycin were included in both media.

Enzyme Assays

Assays of lymphoblast enzymes were usually done on frozen cell pellets for convenience since there was no difference in specific activities if they were assayed in fresh material. Approximately 150 × 10^6 cells were collected from a spinner flask and washed in PBS and 0.25 M sucrose, and the packed cell pellet was stored at −80°C. On the day of assay, the frozen cells were thawed quickly at 37°C and transferred to a 1.0-ml glass homogenizing tube (Bellco Glass, Vineland, N.J.) on ice. The storage tube was rinsed with extraction buffer (0.05 M Tris-HCl, pH 7.5, 10^{-3} M EDTA, 10^{-4} M DTT, 5% glycerol), and this rinse was added to the homogenizing tube. A total of 400 μl of extraction buffer was used if only carboxylase activities were to be assayed, or 100 μl of buffer for synthetase assays, since the latter activity could be detected only in more concentrated cell extracts. After the addition of extraction buffer, 500 μl carbon tetrachloride (CCl_4) was added to the homogenizer and the mixture homogenized with a ground glass pestle until milky colored. The tube was centrifuged at 2,500 rpm at 4°C for 20 min. The aqueous supernatant used for enzyme assays was recovered above a thick lipid interface on top of the CCl_4.

PCC and MCC activities were determined in extracts of lymphoblasts grown in biotin-rich or biotin-restricted medium by the $[H^{14}]CO_3$ fixation assay as described in [10]. PC activity was not detectable over background in lymphoblast extracts from either fresh or frozen cell pellets. Specific activities of the holocarboxylases were defined as the amount of enzyme required to convert 1 nmol of [^{14}C]bicarbonate to acid-nonvolatile form in 1 min, using the appropriate CoA substrate. Protein was determined by the Bio-Rad procedure (Bio-Rad Laboratories, Richmond, Calif.).

Holocarboxylase synthetase activity was assessed by measuring PCC activity in the extracts of biotin-starved lymphoblasts after preincubation in the presence of ATP and biotin. Net increase in PCC activity was taken to represent the effect of synthetase activity. Five μl of a highly concentrated extract of biotin-starved lymphoblasts (containing both apocarboxylase substrate and synthetase enzyme) was combined with 5 μl of preincubation mix to give (final concentrations): 0.3 M Tris-HCl, pH 8.2, 0.05 M reduced glutathione, 22.5 mM $MgCl_2$, 20 mg/l biotin, and 5 mM neutralized ATP. A preincubation period of 60 min at 18°C allowed the conversion of apocarboxylase to holocarboxylase. This temperature was optimal for the synthetase activity determination since higher preincubation temperatures tended to produce a decrease in PCC activity of 12%–15%. Subsequently, PCC activity was measured by adding to the preincubation tube 28 μl of mix containing: 1.78 mM ATP, 0.178% Triton X-100, 0.178 M KCl, and 5.34 mM propionyl CoA. The reaction was initiated by the addition of 12 μl 0.04 M $NaH^{14}CO_3$ (12 mCi/mmol, New England Nuclear, Boston, Mass.), and incubation was continued at 37°C for 15 min. The reaction was stopped by the addition of 50 μl of 1.16 N perchloric acid, and the samples were processed and counted as described for carboxylase assays [10].

Blanks for the synthetase determination were prepared by omitting both ATP and biotin from the preincubation mix. The omission of either ATP or biotin alone gave slightly higher blank values, possibly because of trace amounts of these substrates in the crude extract. Initial (preincubation without ATP or biotin) and final (preincubation with both ATP and biotin) PCC activities are given for all synthetase determinations.

Antibody Absorptions

Anti-PCC antibody raised in rabbits against purified human liver holo-PCC [18] was found to cross-react very potently with both holo- and apo-PCC in normal and patient lymphoblasts (our unpublished observation, 1981). Extracts of normal biotin-starved

lymphoblasts were mixed 1:5 or 1:10 with the antiserum and incubated overnight at 4°C. Antigen-antibody complexes were precipitated by adding protein A bound to formalin-fixed *Staphylococcus aureus* cells (Pansorbin, Calbiochem-Behring, La Jolla, Calif.) [19]. The Pansorbin was incubated with the antiserum-extract mixture for 4 hrs at 4°C. The preparation was centrifuged in an Eppendorf 5412 microfuge for 2 min, and the supernatant containing holocarboxylase synthetase but devoid of apo- or holo-PCC was employed in mixing experiments with biotin-starved patient lymphoblast extracts.

RESULTS

Transformed lymphoblasts were used for these studies because they could be more readily biotin-starved than could fibroblasts. Biotin starvation was used to reduce PCC activity to negligible levels and force the accumulation of apocarboxylases (see below). This could not be achieved with fibroblasts since their carboxylase activities were reduced by only 50% after prolonged culture in the biotin-restricted medium [10]. In contrast, lymphoblasts lost all detectable carboxylase activity in a relatively short period of time in the same medium. This made it possible to compare holocarboxylase synthetase-dependent activation of apo-PCC in extracts of normal and patient cells.

Biotin Starvation

Initially, the carboxylase activities of normal and patient lymphoblasts were compared after growth in biotin-rich and biotin-restricted media to determine if the multiple carboxylase defect was expressed in lymphoblast cultures. In α medium (0.1 mg/l biotin), normal lymphoblasts had PCC activity of 1.89 nmol/min per mg protein (fig. 1*A*) and MCC activity of 2.04 nmol/min per mg protein (fig. 1*B*). Patient lymphoblast enzyme activities were reduced to about 1/3 of normal levels in this medium, with PCC at 0.6 nmol/min per mg and MCC at 0.43 nmol/min per mg. These results contrast with the completely normal PCC and MCC activities obtained for fibroblasts of the same patient grown in α medium (data not shown).

After transfer to biotin-restricted medium, the rate of disappearance of PCC activity was more rapid in patient lymphoblasts than in normal lymphoblasts (fig. 1*A*). While normal cells retained 30% of starting PCC activity after 4 days of biotin starvation and reached background levels after 15 days, patient cells had 18% of PCC activity at 4 days and required only 8 days to reach background (fig. 1*A*). Smaller differences in the rate of decay were found for MCC activity (fig. 1*B*), but disappearance was still slightly faster in the patient than in the normal cells.

Biotin Requirement of Patient Lymphoblasts

Since patient lymphoblasts showed subnormal carboxylase activities even in α medium, we determined the medium biotin concentration at which these cells achieved normal PCC activity. To do this, biotin-starved patient lymphoblasts were transferred to medium containing biotin at 0.1, 1.0, 10, and 100 mg/l and the levels of PCC activity attained were measured (fig. 2). Normal lymphoblasts required less than 3.0 μg/l biotin (contributed by 15% FCS) to maintain normal PCC levels and were not stimulated by higher biotin concentrations (data not

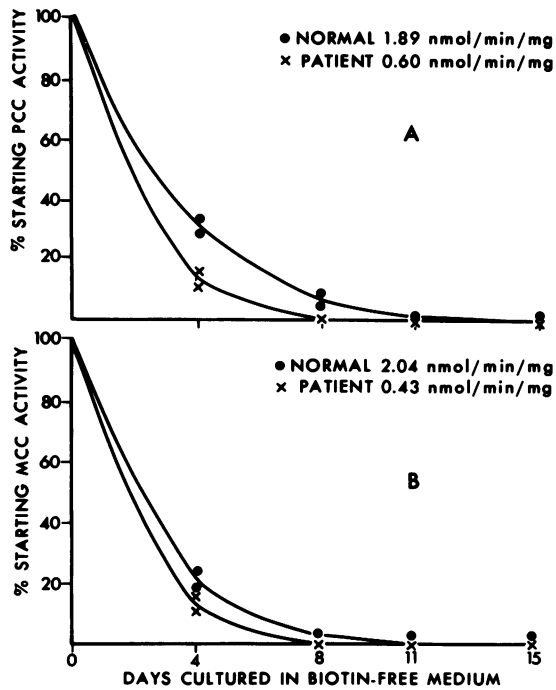

FIG. 1.—PCC (*panel A*) and MCC (*panel B*) activities of lymphoblasts during growth in biotin-depleted medium. Cells were maintained in 200-ml spinner flasks containing α medium (0.1 mg/l biotin) and were transferred at zero time to biotin-restricted medium. Aliquots of 150 ml of each culture were harvested and assayed for PCC and MCC activities at each time point. The remaining 50 ml of each culture were replenished with biotin-restricted medium. Specific activities of PCC (*panel A*) and MCC (*panel B*) for both normal and patient cells at zero time are shown in *upper right portion of each graph*. *Points* shown are duplicate determinations.

shown). As seen in figure 2, the levels of PCC activity in patient lymphoblasts were dependent on the biotin concentration of the medium below 1.0 mg/l. Between 1–5 mg/l biotin was required to achieve normal range activity (fig. 2 and other experiments). Thus, at least a 2,500-fold greater biotin concentration is required to achieve maximal PCC activity in patient than in normal lymphoblasts.

Apocarboxylase Accumulation

The above data showed that it was possible to biotin-starve both patient and normal lymphoblasts to the point where no carboxylase activity was detectable. To develop a detection system for synthetase activity, it was necessary to show whether apocarboxylase accumulated in biotin-starved cells or whether the recovery of carboxylase activity after the addition of biotin required new protein synthesis. Biotin-starved cultures of normal and patient lymphoblasts were transferred to medium containing 30 mg/l biotin with or without 10 μg/ml cycloheximide (which reduces protein synthesis in lymphoblasts to 8% or less) and assayed at various times for PCC and MCC activities (fig. 3). In neither case was

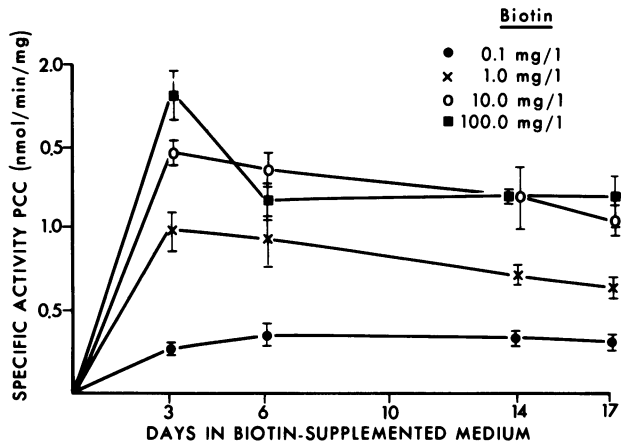

FIG. 2.—PCC activity of patient lymphoblasts during growth in media containing different concentrations of biotin. Cells were grown in biotin-restricted medium to reduce PCC activity to background levels. At zero time, cells were transferred to triplicate 15-ml cultures, 3.3 × 10^6 cells/ml, containing medium supplemented with the indicated biotin concentrations. Aliquots of 11 ml were harvested at approximately 3-day intervals and assayed for PCC activity. The remainder of each culture was replenished with the same medium. Results are expressed as mean PCC activity ± SD of duplicate assays on each of the triplicate cultures.

the restoration of carboxylase activity inhibited by cycloheximide, indicating that no new protein synthesis was required. These results show that apocarboxylase accumulates in both cell lines during biotin starvation and is converted to holocarboxylase on the addition of biotin.

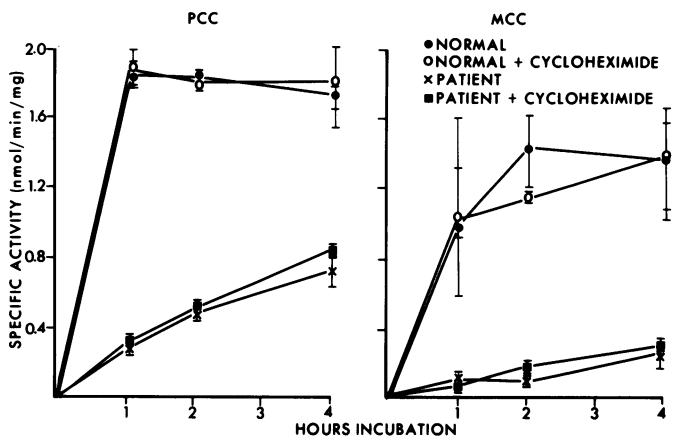

FIG. 3.—Effect of cycloheximide on the whole cell activation of apocarboxylases to holocarboxylases after transfer of biotin-starved lymphoblasts to biotin-rich medium. Cells were grown in biotin-restricted medium until PCC and MCC activities were reduced to background levels. Cells were then transferred to medium containing 30 mg/l biotin with or without 10 μg/ml cycloheximide. One culture of a replicate series, 12 × 10^6 cells/culture, was harvested at each time point and assayed for PCC (*panel A*) and MCC (*panel B*) activities. Results are mean specific activity ± SD of duplicate assays of two independent experiments.

Holocarboxylase Synthetase

The above data show that biotin-starved lymphoblasts must contain both apocarboxylase and synthetase. If biotin and ATP were added to extracts of these cells, the synthetase would be expected to catalyze the formation of the holocarboxylase, with consequent PCC activity. The increase in PCC activity should provide a measure of the activity of the synthetase enzyme. The data in figure 4 indicate the validity of this approach.

Figure 4 shows that the net PCC activity derived from apo-PCC conversion was linear with the amount of extract protein added above 90 μg (9 mg/ml). The nonlinearity below 9 mg/ml presumably was due to excessive dilution of the extract preventing the efficient combination of apocarboxylase and synthetase. In different experiments, the lower limit of linearity varied between 5–10 mg/ml protein. The assay was linear with time for at least 60 min (data not shown). The specific activity of PCC achieved over the linear range of protein concentration was 0.22 nmol/min per mg in this experiment. This is equivalent to 18.9% of the activity obtained in the extract of normal lymphoblasts grown in biotin-rich medium (1.17 nmol/min per mg).

Table 1 shows the results of a synthetase-dependent activation experiment carried out in lymphoblast extracts of the biotin-responsive patient and the control. The extract of normal biotin-starved lymphoblasts showed a net increase in PCC activity of 0.29 nmol/min per mg at a protein concentration of 11.5 mg/ml, an activation to 26% of maximal levels. The extract of biotin-starved patient cells showed no appreciable PCC activity when preincubated with ATP and biotin at any protein concentration (table 1). These results strongly suggest that the patient cells contain a defect in their ability to activate apocarboxylase.

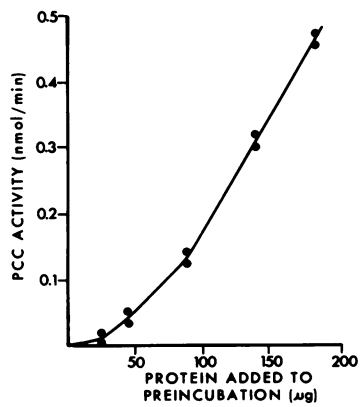

FIG. 4.—Dependence of holocarboxylase synthetase-dependent PCC activity on protein concentration. Normal lymphoblasts were grown in biotin-restricted medium until PCC activity was reduced to approximately background levels, 0.03 nmol/min/mg in this experiment. Cells were harvested and assayed for biotin and ATP-dependent activation of apo-PCC as described in MATERIALS AND METHODS. Values shown are duplicate determinations of the net PCC activity in the indicated amount of cell extract after preincubation with ATP and biotin.

TABLE 1

HOLOCARBOXYLASE SYNTHETASE-DEPENDENT APO-PCC ACTIVATION IN EXTRACTS OF BIOTIN-STARVED NORMAL AND PATIENT LYMPHOBLASTS

Lymphoblast strain	Growth condition*: biotin in culture	Protein in preincubation (mg/ml)	Initial PCC† activity (nmol/min/mg)	Final PCC activity (nmol/min/mg)	Net (synthetase-dependent) PCC activity (nmol/min/mg)
Normal.....	+	13.8	1.12‡	0.96	...
Patient	+	7.6	0.61	0.63	...
Normal.....	−	11.5	0.02	0.31	0.29
	−	7.9	0.01	0.13	0.12
Patient	−	13.0	< 0.01	0.01	...
	−	7.4	< 0.01	< 0.01	...

* +, lymphoblasts cultured in α medium (0.1 mg/l biotin); −, biotin-restricted medium.

† "Initial" PCC activity is activity after preincubation without ATP and biotin; "final" PCC activity, after preincubation with ATP and biotin.

‡ Values shown are the average of duplicate determinations.

Extracts of normal cells cultured in the biotin-rich α medium had PCC specific activity not significantly affected by preincubation with or without ATP and biotin, indicating that these cells were maximally activated. An extract of patient lymphoblasts cultured in α medium showed no increase in PCC activity after preincubation with ATP and biotin, even though maximal activation had not been achieved during growth in the biotin-rich medium. The biotin concentration in the preincubation mix was 20 mg/l, well in excess of the concentration required for recovery of normal levels of carboxylase activity in whole cells. Increasing the biotin concentration in the preincubation mix up to 250 mg/l had no effect on patient synthetase activity.

Mixing Experiments

The usefulness of the above synthetase determination is somewhat limited because the total activity in the preincubated extracts is dependent on two factors, the synthetase and the apoenzyme. It was therefore determined whether the synthetase of normal cells could be used in mixing experiments to catalyze the activation of the apo-PCC in a patient cell extract. The source of normal synthetase was an extract of normal biotin-starved lymphoblasts from which apo-PCC and any residual holo-PCC had been removed with anti-PCC antibody. Before treatment with antibody, 3 μl of the normal extract exhibited 13.0 pmol/min synthetase-dependent PCC activity (line 2, table 2). This activity disappeared if the normal extract was absorbed with anti-PCC before the preincubation step. Extracts from biotin-starved patient cells alone showed no biotin- and ATP-stimulated PCC activity (line 1, table 2), but when patient cell extracts were combined in equal amounts with the antibody-treated normal extract, very significant increases were observed in synthetase-dependent PCC activity (table 2). A net PCC activity of 3.38 pmol/min was achieved in experiment A and 5.03 pmol/min in experiment B, representing 14.4-fold and 20.5-fold increases, respectively. These results provide

TABLE 2

ACTIVATION OF APO-PCC IN PATIENT LYMPHOBLAST EXTRACTS BY APO-PCC-FREE NORMAL LYMPHOBLAST EXTRACTS

Lymphoblast extract	Initial* PCC activity (nmol/min × 10^3)	Final PCC activity (nmol/min × 10^3)	Net (synthetase-dependent) PCC activity (nmol/min × 10^3)	Increase†
Untreated normal‡	1.97 ± 0.83	14.97 ± 0.37	13.00 ± 0.52	...
Patient§	0.46 ± 0.01‖	0.70 ± 0.12	0.24 ± 0.07	...
Experiment A#:				
Treated normal	0.15 ± 0.03	0.17 ± 0.04	0.02 ± 0.03	...
Patient + treated normal	0.55 ± 0.03	4.43 ± 0.46	3.38 ± 0.23	14.4×
Patient + treated normal, diluted	0.73 ± 0.02	3.10 ± 0.33	2.37 ± 0.19	8.4×
Experiment B:				
Treated normal	0.14 ± 0.02	0.14 ± 0.04	0	...
Patient + treated normal	0.61 ± 0.06	5.64 ± 0.91	5.03 ± 0.53	20.5×
Patient + treated normal, diluted	0.68 ± 0.04	4.38 ± 0.75	3.70 ± 0.43	14.9×

* "Initial" and "final" PCC activities are as defined in footnote†, table 1.

† No. fold increase over the expected value in synthetase-dependent PCC activity in the patient extract.

‡ Original normal biotin-starved lymphoblast extract before absorption with anti-PCC antibody; preincubation protein concentration was 118 μg/3λ.

§ Extract of biotin-starved patient lymphoblasts; protein concentration was 111 μg/3λ in the preincubation.

‖ Values are the mean PCC activity ± SD of triplicate assays. A blank equivalent to 3.7×10^{-4} nmol/min has been subtracted from each value for counts present in the absence of propionyl-CoA. 1×10^{-3} nmol/min = 421 dpm.

In experiment A, 200λ biotin-starved normal lymphoblast extract was absorbed with 20λ anti-PCC (see MATERIALS AND METHODS) to give the "treated" normal extract; this extract was then diluted 1:1 with buffer to give results in line 5. Experiment B was done with same extracts except 100λ of the normal extract was absorbed with 20λ anti-PCC; this preparation was also tested at a 1:1 dilution (line 8).

strong evidence that the normal extract retained synthetase activity after apoenzyme absorption and that the normal synthetase could activate patient apoenzyme, while the endogenous patient synthetase could not.

DISCUSSION

Evidence that lymphoblasts from patient J. T. expressed the multiple carboxylase disorder include the following: (1) the presence of only about one-third of normal PCC and MCC activities after culture in standard α medium, (2) a requirement for a large increase in medium biotin concentration to produce normal levels of carboxylase activities, and (3) a slightly increased rate of biotin starvation.

When the patient lymphoblasts were assayed for apo-PCC activation, they proved to be devoid of holocarboxylase synthetase activity. Since our assay made use of endogenous apo-PCC as the substrate for the synthetase, we could not distinguish directly between a defective synthetase and an altered apocarboxylase substrate. However, there are several indications that we are dealing with a defect of the synthetase. First, in mixed extract experiments, normal cell holocarboxylase synthetase was able to activate patient apo-PCC as much as 20-fold over starting levels. Second, no new protein synthesis was required for the recovery of carboxylase activity either in the lymphoblasts of patient J. T. or in the fibroblasts of another *bio* patient [20]. Third, McKeon et al. [21] have used immunotitration experiments to show that normal amounts of PCC protein are present in extracts of biotin-starved *bio* fibroblasts. We have confirmed this result in preliminary experiments for lymphoblasts of patient J. T. Finally, fibroblasts of the *bio* complementation group have been shown to complement cells from patients with single defects of the PCC or PC apocarboxylase [10, 13].

We therefore believe that the apoenzyme in cells of the *bio* patients is normal both in quality and quantity, and that the defect in apo-PCC activation is caused by a deficiency of holocarboxylase synthetase activity. It remains unclear whether single or multiple synthetases are involved in the maintenance of the three mitochondrial carboxylases (PCC, MCC, PC) and the sole cytosolic carboxylase, acetyl CoA carboxylase (ACC). Recently, Feldman and Wolf [22] reported that ACC activity is also deficient in fibroblasts of a *bio* patient. This would suggest that a single synthetase is responsible for biotinylating all four apocarboxylases.

The biotin-dependent recovery of carboxylase activities occurred in the patient in vivo and in whole cell experiments with cultured cells, but not in cell extracts. This result could be explained if the mutant synthetase were more labile than the normal enzyme in the broken cell preparation. Because whole cell experiments have shown that increased amounts of biotin are required for patient cells to maintain normal carboxylase activities, we suggest that the biotin-responsiveness might be due to the presence of a holocarboxylase synthetase with a decreased affinity for biotin.

NOTE ADDED IN PROOF: While this manuscript was in press, Burri et al. [23] reported that the fibroblasts of another patient from the *bio* complementation group had a

holocarboxylase synthetase activity with a reduced V_{max} and a K_m for biotin 60× normal.

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