Am J Hum Genet 30:455–464, 1978

Biochemical Differences between Mutant Propionyl-CoA Carboxylases from Two Complementation Groups

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INTRODUCTION

Propionic acidemia is a recessively inherited disorder of organic acid metabolism caused by a deficiency of propionyl-CoA carboxylase (PCC; E.C.6.4.1.3) activity [1]. Leukocytes and cultured fibroblasts from affected patients uniformly retain some detectable carboxylase activity which shows distinctly increased thermolability [2]. This suggests that the deficiency state reflects structural alterations in the PCC enzyme itself [2]. Recently Gravel et al. [3] conducted genetic complementation tests in heterokaryons formed from pairwise crosses of numerous PCC deficient fibroblast cell lines, using an autoradiographic method which detects [1-^{14}C] propionate utilization by intact cells in situ. They demonstrated two major complementation groups among PCC deficient lines, which were designated the A and C mutant classes. The identification of two complementation groups implies the existence of two different mutations leading to PCC deficiency, but does not distinguish between interallelic and intergenic complementation.

In this report, we describe some biochemical features of the residual PCC activity in these two major complementation groups. Based on these findings, we propose two possible models for the structure of normal and mutant PCC in human cells.

MATERIALS AND METHODS

Cultured skin fibroblasts were propagated from seven children with PCC deficiency and from four controls. Three of the PCC deficient lines belonged to the A complementation group [A. G. (49); R. H. (117); and Baby Girl V (318)] and four to the C complementation group [G. S. (94); P. C. (148); N. M. (269); and K. M. (270)] as designated by Gravel et al. [3]. Numbers in parentheses refer to cell line designations in our laboratory. Clinical information about these

Received March 8, 1978; revised April 26, 1978.

This work was supported in part by a research grant from the National Institutes of Health (AM 12579) and was presented in part at the 28th Annual Meeting of The American Society of Human Genetics, October 1977, San Diego, California. B. W. is a recipient of a traineeship (grant GM 00077) from the National Institutes of Health.

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patients has been reported previously [2]. Control and mutant fibroblasts were grown in Dulbecco's modified minimal essential medium supplemented with 10% fetal calf serum, nonessential amino acids, and kanamycin (100 μg/ml). Cells were harvested with 0.25% trypsin-EDTA, washed twice with normal saline, and centrifuged at 100 *g* for 5 min. Cell pellets thus obtained were stored frozen at −68°C for up to 2 days; such pellets of both control and mutant cells could be frozen for up to 2 weeks without loss of PCC activity. Pellets were thawed at room temperature for 15 min and suspended in 50 mM Tris-HCl buffer, pH 8.0 with 0.5% Triton X-100 (Sigma, St. Louis, Mo.) to disrupt the cells. The suspension was centrifuged at 100 *g* for 5 min to remove the cellular debris. The supernatants were dialyzed twice against 1,000 ml of 50 mM Tris-HCl buffer, pH 8.0, containing 1 mM reduced glutathione (Sigma), and were used directly as a source of enzyme.

PCC activity was assayed by a modification of a procedure described previously [4]. The standard incubation mixture contained (in a final volume of 100 μl): 50 mM Tris-HCl, pH 8.0; 5 mM reduced glutathione; 2 mM sodium adenosine triphosphate (ATP) [Sigma]; 100 mM KCl; 10 mM $MgCl_2$; 10 mM sodium [^{14}C]bicarbonate (New England Nuclear, Boston, Mass.; final specific activity 12 mCi mmol); 3 mM propionyl-CoA and cell extract (0.2–0.3 mg protein). Propionyl-CoA was prepared from propionyl anhydride (Eastman Organic Chemicals, Rochester, N.Y.) and coenzyme A (P-L Biochemicals, Milwaukee, Wis.) by the method of Flavin and Ochoa [5]. Each assay was performed in duplicate with a blank control omitting the propionyl-CoA. The mixture was incubated at 37°C for 15 min, and the reaction terminated by addition of 50 μl 10% trichloracetic acid. Following centrifugation at 100 *g* for 15 min, a 0.1 ml aliquot of the supernatant was placed in a scintillation vial and dried slowly under a heat lamp. The residue was dissolved in 0.3 ml water. Four ml of Aquasol (New England Nuclear) was added, and the samples were counted in a Packard liquid scintillation spectrometer. Protein concentrations were determined in a separate aliquot of the supernatant by the method of Lowry et al. [6], using bovine serum albumin as the standard. Enzyme specific activity is expressed as pmol $^{14}CO_2$ fixed per min/mg protein. The velocity of carboxylase activity was linear to 30 min with up to 0.4 mg cell extract protein.

p-Hydroxymercuribenzoate, *d*-biotin, and α-aminoisobutyrate (AIB) were purchased from Sigma, and avidin was obtained from Worthington (Freehold, N.Y.).

RESULTS

Residual PCC Activity

PCC activity in the three mutant lines of complementation group A ranged from four to 47 pmol per min/mg protein and in the four lines from complementation group C from three to 68 pmol per min/mg protein (control activity ± SD: 845 ± 150 pmol/min/mg). No mutant line studied exhibited complete absence of activity, and no consistent difference in residual activity was observed between mutant lines of the A and C complementation groups.

Kinetic Parameters and Sulfhydryl Inhibition

The K_m values for each of the enzyme's substrates (propionyl-CoA, bicarbonate, ATP, and magnesium) were similar in control, group A, and group C extracts (table 1). Since PCC is known to contain an essential sulfhydryl group in its active site [7], we examined the effect of incubating cell extracts at 37°C for 10 min with *p*-hydroxymercuribenzoate, a powerful sulfhydryl inhibitor. PCC activity from control lines and from both mutant classes was inhibited completely.

TABLE 1

AFFINITY OF MAJOR SUBSTRATES FOR PROPIONYL-CoA CARBOXYLASE

SUBSTRATE	K_m (mM)		
	Controls	Group A	Group C
Propionyl-CoA	0.5 ± 0.3	0.7 ± 0.5	0.6 ± 0.4
Bicarbonate	2.1 ± 0.8	1.6 ± 1.0	2.2 ± 0.4
ATP	0.8 ± 0.4	1.0 ± 0.4	0.9 ± 0.5
Magnesium	2.1 ± 0.3	2.1 ± 0.6	1.9 ± 0.8

NOTE.—K_m values (mean ± 1 SD) were determined from plots of 1/v vs. 1/substrate in both control (four lines) and mutant (three group A and 4 group C lines) cell extracts. The ranges of concentrations of the various substrates examined were: propionyl-CoA, 0.1–3.0 mM; $NaHCO_3$, 0.05–2.0 mM; sodium ATP, 0.1–4.0 mM; $MgCl_2$, 0.125–15.0 mM.

Thermostability of PCC

The first suggestion of interclass differences came from thermostability studies with control and mutant extracts (fig. 1). Cell extracts were incubated at 45°C for intervals up to 15 min and then assayed at 37°C for residual PCC activity. Control enzyme was distinctly more stable than that of either mutant class; after 10 min at 45°C the mean percent of initial PCC activity in controls was 83%, compared to values of 50% in group C extracts and 17% in those from group A. Furthermore, PCC activity in mutant cells belonging to complementation group A decayed distinctly more rapidly than did that in cells from group C.

Biotin-Avidin Interactions

Since PCC activity depends on the presence of biotin covalently bound to the enzyme protein [8] and since avidin, a protein which binds free and bound biotin very tightly, is capable of totally inactivating all biotin-dependent enzymes [9], we examined the effect of addition of excess avidin on PCC activity from mutant and control cells. Avidin (0.01 to 0.5 U) was incubated with cell extracts at 37°C for 15 min. The remainder of the reaction mixture was then added and PCC activity assayed. We observed virtually complete loss of PCC activity in both control and mutant extracts (table 2), suggesting that PCC from the mutant cell lines studied retains bound biotin. A second experiment examining biotin-avidin interaction involved the reactivation of avidin-inactivated PCC by the addition of excess biotin (table 2). PCC activity of the various extracts was titrated with increments of 0.025 U of avidin until no activity remained. Excess biotin (4 μg) was added, and PCC activity was again assayed after incubation with biotin for 2–120 min. Maximal restoration of PCC activity by excess biotin was reached after 2 min in all cell lines. The range of restoration of initial PCC activity for control lines was 10%–20%; that for group C cells, 30%–50%; and for group A cells, always 100%.

Potassium Activation of PCC

PCC activity in pig heart has been demonstrated previously to be elevated six to 11-fold by the addition of monovalent cations such as potassium [10, 11]. Potassium

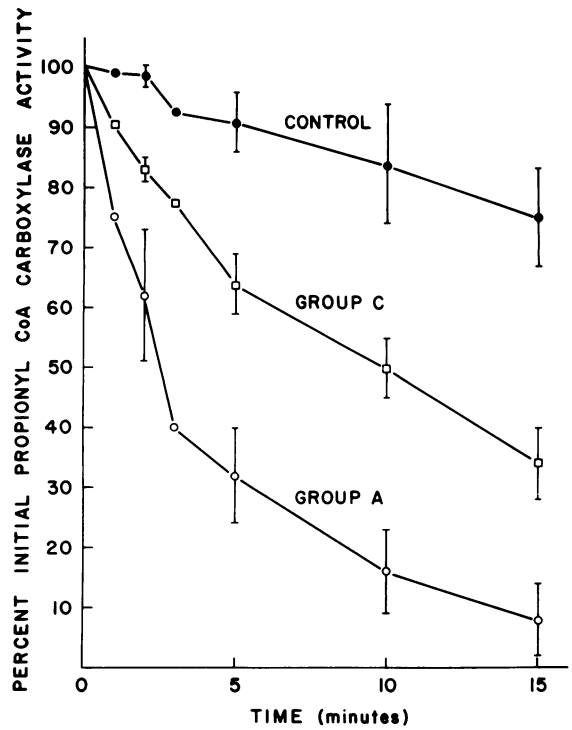

FIG. 1.—Thermostability of control and mutant PCC. Extracts of control (●), group C (□), and group A (○) cells were incubated at 45°C for intervals up to 15 min as noted on the abscissa. After each interval, residual PCC activity was measured and is expressed on the ordinate as the mean percent of PCC activity without incubation at 45°C. *Vertical bars* denote 1 standard deviation above and below the mean for duplicate observations on extracts from four control, four group C, and three group A lines. Values without vertical bars denote duplicate observations on a single representative line from each class.

activation of pig heart PCC activity results in a two to threefold lowering of the K_m for bicarbonate with a concomitant change in entropy, suggesting that potassium induces a conformational change in the PCC molecule [12]. We, therefore, examined the kinetics of potassium activation of control and mutant PCC. The apparent K_m of potassium activation for PCC in control lines was 9.0 ± 1.0 mM (fig. 2); the apparent K_m for

TABLE 2

RESTORATION OF AVIDIN-INACTIVATED PROPIONYL-COA CARBOXYLASE ACTIVITY BY BIOTIN IN CONTROL AND MUTANT LINES

Experimental Conditions	Line 82 (Control)	Line 49 (Complementation Group A)	Line 269 (Complementation Group C)
Initial activity	815	42	67
Activity after inactivation with excess avidin	10	0	3
Activity after addition of excess biotin	120	42	31
Restoration of activity	14%	100%	46%

NOTE.—Data are from one of four representative experiments. Activity = pmol/min/mg protein.

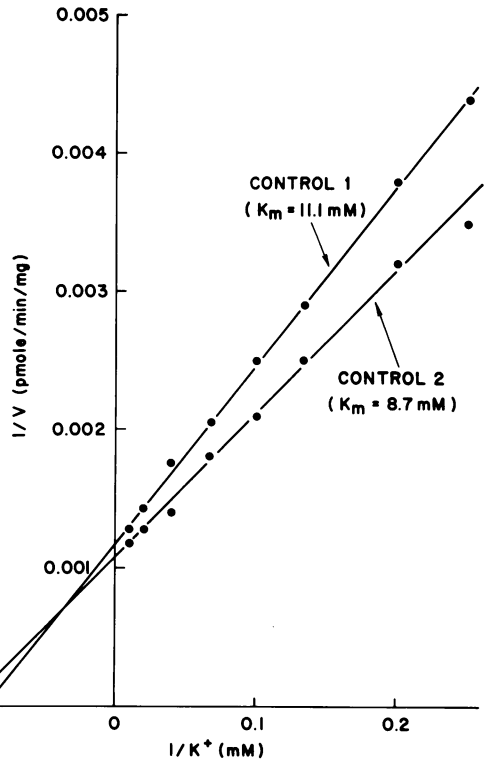

FIG. 2.—Apparent K_m values of potassium activation for PCC from two representative control lines. K_m values were determined from least square regression analyses.

mutant PCC from group C cell lines was similar (9.5 ± 0.7 mM) [fig. 3]. In contrast, the apparent K_m of potassium activation for mutant PCC from group A lines was found to be four to fivefold greater (41.1 ± 9.0 mM) [fig. 3], suggesting a conformational alteration in PCC from the group A cells.

Effects of α-Aminoisobutyrate

We observed that high concentrations of glycine and other short chain aliphatic amino acids such as alanine and serine enhance mutant, but not control, PCC activity. The glycine analogue, AIB, was employed most extensively in these experiments because it is as effective as glycine, but is not metabolized. AIB (200 mM) was incubated with the various extracts at 37°C for 20 min prior to assaying PCC activity under standard conditions. Figure 4 illustrates that PCC activity from group A cells was stimulated three and a half to eightfold by AIB. PCC activity from group C cells was also stimulated, but never more than twofold.

Thermostability studies at 45°C revealed that AIB nonspecifically stabilized both control and mutant PCC (data not shown). AIB had no effect on the K_m values and Hill coefficients for propionyl-CoA, bicarbonate, and potassium in group A cells. However, the K_m for ATP in the presence of AIB (0.15 mM) in group A cells was about

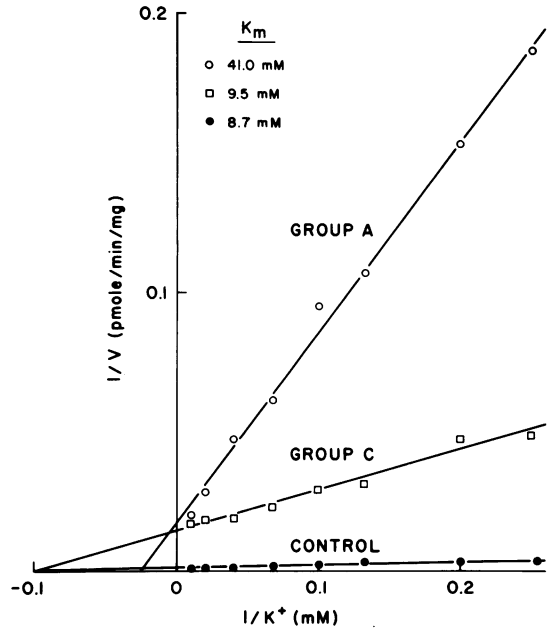

FIG. 3.—Apparent K_m values of potassium activation for PCC from representative mutant lines compared to a control line. Representative control (●), group A (○), and group C (□) lines. Note the change in ordinate scale from that in figure 2.

one-fourth the K_m without AIB (0.65 mM). These results suggest that AIB may enhance or stabilize ATP binding in the A mutants, thereby leading to the increase in PCC activity noted in figure 4.

DISCUSSION

The biochemical studies presented here suggest that propionyl-CoA carboxylases from the two major mutant complementation groups reflect different structural gene mutations. Our data suggest that the mutant enzymes contain biotin, possess an intact sulfhydryl group in the active site, and exhibit Hill coefficients and K_m values for all major substrates within the range observed for control lines. However, results of experiments measuring thermostability, avidin-biotin interactions, potassium activation, and AIB stimulation indicate that the mutant enzymes from the two complementation groups differ from one another and from control PCC.

The estimated K_m values for propionyl-CoA and bicarbonate agree well with values reported for highly purified or crystalline mammalian PCC [8, 14]. The K_m for ATP, however, although similar for control and mutant PCC in this study, is slightly higher than the value reported for crystalline mammalian PCC. Since our values were obtained using crude extracts, higher K_m values may reflect utilization of ATP by other enzymes during the assay. Alternatively, these data may, in fact, represent a real difference between human and other mammalian PCCs.

There are two major possible explanations for those observations concerning the reactivation of avidin-inactivated PCC by excess biotin. First, the binding of avidin to

the biotin on PCC may be weaker in mutant cells than in controls. Since the association constants of avidin for both free and protein-bound biotin are similar at $\sim 10^{-15}$ M [13], the binding by avidin of biotin on PCC is essentially irreversible in control cells. If the biotin is positioned within a recess of the active site which is modified by the mutations in group A and group C cells, however, biotin-avidin binding in mutant PCC may be considerably weaker. Perhaps, for example, a structural alteration in the mutant enzyme makes the bound biotin less accessible to avidin and, thereby, allows for greater complexing of avidin with added free biotin. The second possible explanation does not invoke a weaker association between bound biotin and avidin but assumes that excess free biotin within the active site can be carboxylated [15] and can transfer the carboxyl group to propionyl-CoA without removal of avidin. Regardless of the mechanism, the restoration of PCC activity is greater for group A than for group C mutants, thereby distinguishing between the two complementation groups.

Hsia et al. [2] determined that partially purified, normal human PCC from skin fibroblast extracts has a molecular weight of approximately 700,000. Crystalline pig heart PCC has a similar molecular weight and contains four biotin molecules per molecule of native PCC [8]. Ultracentrifugation studies of crystalline pig heart PCC treated with 7 M urea revealed dissociation of PCC into a single inactive species with a molecular weight of 175,000 [8], suggesting that animal PCC is a tetramer with one biotin molecule per protomer [8].

The number and character of polypeptide subunits in a mammalian PCC protomer is unknown. Based on the defined polypeptide composition of other prokaryotic and eukaryotic carboxylases, several subunit structures are possible [16, 17]. First, the

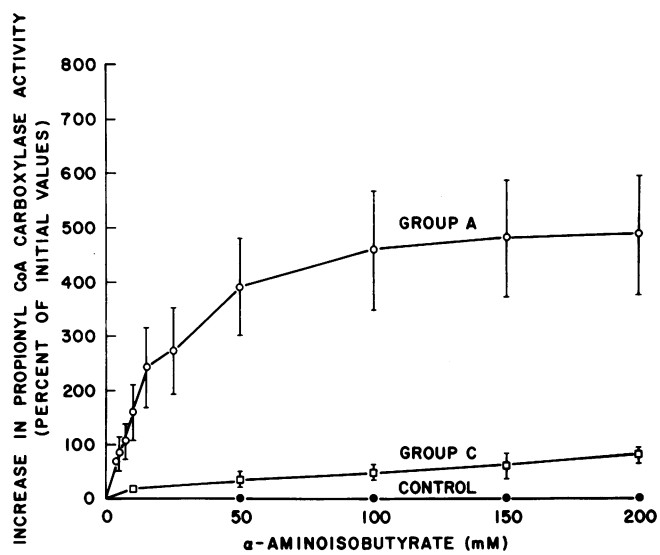

FIG. 4.—Effect of AIB (200 mM) on PCC activity in control (●), group A (○), and group C (□) cell extracts. Increase in PCC activity is expressed as percent (± 1 SD) of activity without AIB. Data were obtained from studies with four control, three group A, and four group C lines. Values without standard deviation represent single observations. See text for additional details.

protomer may be composed of a single large polypeptide, such as the 120,000–130,000 one found in pyruvate carboxylase purified from several eukaryotes [18]. However, in their survey of 99 human enzymes, Hopkinson et al. [19] found none with a subunit molecular weight greater than 116,000. Hence, a subunit size of 175,000 for human PCC might be deemed unlikely. Second, the protomer could be composed of two or more identical subunits. This would satisfy the size criterion just mentioned, but presents a more formidable objection, namely, how to explain the existence of only one biotin molecule per protomer. A third, and leading possibility is that the PCC protomer consists of at least two nonidentical subunits. In fact, nonidentical subunits have been reported in the protomer of a bacterial PCC [20] and of another closely related bacterial enzyme, β-methylcrotonyl-CoA carboxylase [21]. In the latter case, only one of the two subunits contained biotin.

Until the subunit structure of the human PCC protomer is known, it will be impossible to determine whether the mutations responsible for the two major PCC complementation groups occur within the same or different structural gene loci. Our results can be interpreted equally well using either single gene or multigene models for the PCC protomer (fig. 5). The single gene model requires regions of distinct function; the multigene model ascribes these different functions to different subunits. For convenience, we will first interpret the chemical differences observed in terms of the multigene model.

In this model, one subunit, α, is the biotin carboxylation subunit which catalyzes the addition of a carboxyl group from bicarbonate to the covalently bound biotin [17, 22]. This reaction requires ATP and magnesium [22]. Since potassium is known to alter the binding of bicarbonate, and since bicarbonate is located on the biotin carboxylation subunit, the potassium site is almost surely on this subunit as well. A second subunit, β, is the carboxyl transferase subunit which catalyzes the transfer of the carboxyl group

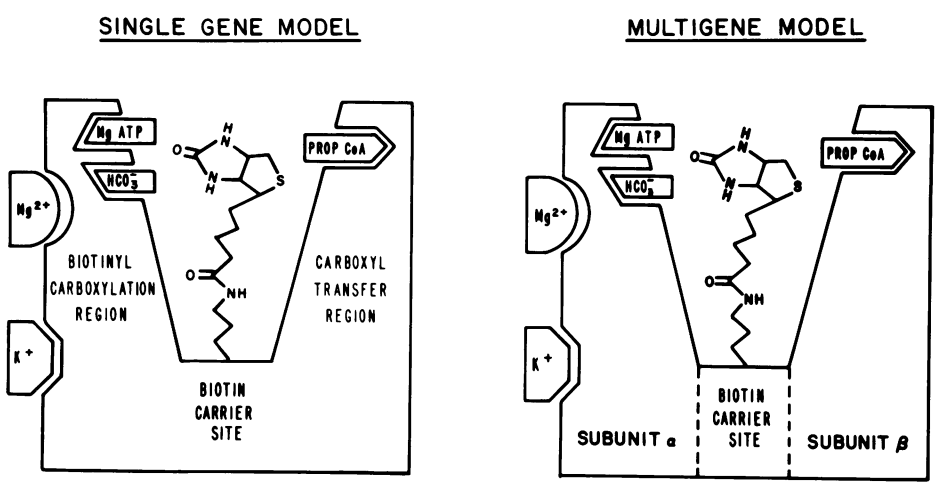

FIG. 5.—Possible models of human PCC protomer. In the single gene model (*left*), the protomer is composed of single polypeptide chain with regions of discrete function. In the multigene model (*right*), the protomer is composed of at least two different subunits, each subserving a specific function. The biotin carrier site could be part of the α or β subunit; it could also be a discrete subunit.

from biotin to propionyl-CoA producing methylmalonyl-CoA [17, 22]. Divalent cations and ATP are not required for this reaction [23, 24]. Finally, the enzyme contains a biotin carrying portion which may be a separate polypeptide but is more likely part of either the α or β subunit [16, 17]. Since PCC from mutant complementation group A showed both reduced affinity for potassium and the greatest response to amino acids known to alter ATP binding, it is attractive to propose that the mutation in this class involves the α subunit. If the complementation observed by Gravel et al. between A and C mutants is intergenic, then it follows that the C mutants reflect defects in the β subunit. If, on the other hand, the protomer is the product of a single gene, then the complementation between A and C mutants must be interallelic.

In keeping with nomenclature used to classify mutant strains in bacteria, we propose that the generic abbreviation *pcc* be used for all mutations of the structural gene locus or loci coding for the PCC polypeptide(s). The suffix A, B, C, etc. would then identify the individual complementation groups. Thus, we propose that complementation group A be called *pcc* A and complementation group C, *pcc* C, etc.

SUMMARY

We examined several biochemical parameters of propionyl-CoA carboxylase (PCC) activity in fibroblast extracts from PCC deficient patients belonging to the two major genetic complementation groups (A and C). Three representative fibroblast lines from the A complementation group and four from the C complementation group were compared with each other and with four control lines. Mutant enzymes from both groups contain biotin, possess a sulfhydryl group in their active sites, and exhibit Hill coefficients and K_m values for all major substrates within the control range. The following chemical differences were observed between the complementation groups: (1) at 45°C PCC from the A complementation group decayed distinctly more rapidly than did the group C enzyme, and both mutant PCCs were significantly more thermolabile than control enzyme; (2) potassium activated PCC from both mutant classes and from controls, but group A PCC had a fivefold lower affinity for this monovalent cation; (3) α-aminoisobutyrate (AIB) enhanced mutant but not control PCC activity, this effect being greater in enzyme from group A than group C lines; (4) following total inhibition of PCC activity by avidin, excess biotin restored 100% of activity in group A lines and 30%–50% in group C lines, compared to only 10%–20% in controls. Based on the known structures of prokaryotic and eukaryotic carboxylases, we propose that mutant PCC from complementation group A is structurally altered in the region of the enzyme containing the bicarbonate and adenosine triphosphate (ATP) binding sites. We propose further that mutant PCC from complementation group C is modified in a region distinct from that affected in group A mutants, perhaps in that portion containing the propionyl-CoA binding site. It is not yet possible to determine if the mutations responsible for these two classes of structurally altered PCCs are allelic or nonallelic.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the advice and suggestions of K. J. Scully, F. Kalousek, K. Tanaka, and H. F. Willard, and the technical assistance of R. J. Blunden.

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