Acyl-Coenzyme A Carboxylases. Homologous 3-Methylcrotonyl-CoA and Geranyl-CoA Carboxylases from *Pseudomonas citronellolis*†

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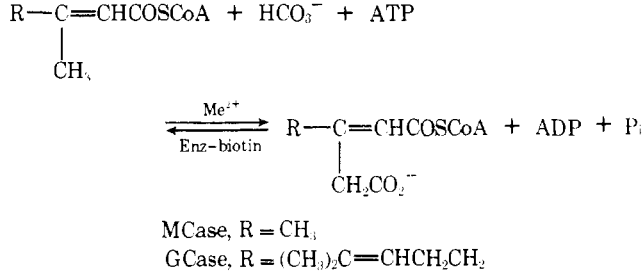
ABSTRACT: 3-Methylcrotonyl-CoA carboxylase (MCase) and geranyl-CoA carboxylase (GCase) have been separately induced and purified from *Pseudomonas citronellolis*. These two enzymes, that catalyze analogous carboxylations at the β-methyl group of their respective acyl-CoA substrates, exhibited identical purification behavior. Both enzymes have approximate molecular weights of 520 000–580 000 as measured (a) by gel filtration and (b) from exclusion limits by polyacrylamide gel electrophoresis at pH 7.9. In the latter procedure each [^{3}H]biotin-labeled enzyme revealed a single identically migrating protein band with coincident [^{3}H]biotin and enzymatic activity. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate revealed that each enzyme contains different subunits, including a smaller biotin-free subunit (A subunit) and a larger biotin-containing subunit (B subunit). Molecular weights determined by electrophoresis in sodium dodecyl sulfate at pH 7.2 were 63 000 and 73 000 for the MCase subunits, and 63 000 and 75 000 for the GCase subunits. Electrophoresis of a mixture of MCase and GCase in sodium dodecyl sulfate at pH 8.9 resolved their respective A and B subunits, demonstrating that these two enzymes do not share common subunits. The biotin contents of the two enzymes were very similar ranging from 148 000 to 157 000 g of protein per mol of biotin. These biotin prosthetic groups of the two complexes were not equally accessible to the inhibitor avidin, with GCase being more strongly inhibited. While both enzymes catalyzed the model reaction, ATP-dependent carboxylation of *d*-biotin, MCase catalyzed it at only 0.5–1% the rate of 3-methylcrotonyl-CoA carboxylation, and GCase at 3–5% the rate of geranyl-CoA carboxylation. It is concluded that these two enzyme complexes contain very similar but not identical substructures, probably with an A_4B_4 stoichiometry.

The enzyme 3-methylcrotonyl coenzyme A carboxylase (MCase)[1] has been described as part of the leucine catabolic pathway in liver mitochondria and various bacteria (reviewed by Alberts and Vagelos, 1972). MCase catalyzes an ATP and divalent metal ion dependent carboxylation of a β-methyl group of 3-methylcrotonyl-CoA as shown in Scheme I and is one of a class of biotin-dependent, acyl-CoA carboxylases. The enzyme from *Achromobacter* has been crystallized and extensively characterized (Apitz-Castro et al., 1970; Schiele et al., 1975; Schiele and Stürzer, 1975).

Seubert et al. (1963) have described and partially characterized a related acyl-CoA carboxylase, GCase, which catalyzes an analogous carboxylation as shown in Scheme I. GCase is an inducible enzyme found in *Pseudomonas citronellolis* grown on monoterpenes such as citronellol (Seubert et al., 1963), or citronellic or geranoic acid (Hector and Fall, 1976a). We have recently shown that extracts of *P. citronellolis* grown on geranoic acid as sole carbon source contain both GCase and MCase activities, and that these two activities probably reside on the same enzyme, GCase. A distinct MCase, that doesn't carboxylate geranyl-CoA appreciably, is induced in *P. citronellolis* grown on leucine or isovalerate (Hector and Fall, 1976a,b).

This communication describes the purification of MCase and GCase and presents evidence that they are structurally similar, but distinct, enzyme complexes.

SCHEME I



Experimental Procedure

Materials. The sources of most of the materials used have been previously described (Hector and Fall, 1976a,b); others were obtained as indicated below or were reagant grade. Protein standards for molecular weight determinations were obtained from Sigma.

A mixture of *cis*- and *trans*-geranoic acid was synthesized from citral (Pfaltz and Bauer) by Ag_2O oxidation as described by Shamma and Rodriquez (1968). To prepare *cis*-geranoic acid, the geranoic acid mixture was converted to the methyl ester derivatives with diazomethane, and the *cis*- and *trans*-geranoic acid methyl esters were separated by thin-layer chromatography on preparative silica gel plates developed twice with diethyl ether–hexane (1:9). The respective cis and trans isomers were eluted from the silica gel with diethyl ether and saponified to yield the respective acids. Citronellic acid was synthesized from citronellal (Pfaltz and Bauer) by the procedure of Katsura et al. (1963).

3-Methylcrotonyl-CoA was prepared from 3-methylcrotonic acid by the mixed anhydride method described by Stadtman (1956); *cis*-geranyl-CoA was prepared from *cis*-geranoic acid by a similar procedure, except that the $KHCO_3$ solution was

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[1] Abbreviations used: MCase, 3-methylcrotonyl-coenzyme A carboxylase; GCase, geranyl-coenzyme A carboxylase; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; BSA, bovine serum albumin; P_i, inorganic phosphate.

prepared in 50% (v/v) tetrahydrofuran. The CoA thioesters were quantitated by the hydroxamate method of Shapiro (1953), using succinic anhydride as a standard.

Analytical Techniques. MCase and GCase assays were carried out as previously described (Hector and Fall, 1976a). The [^{14}C]CO_2 fixation assay was used for all fractions prior to the gel filtration step, after which the spectral assay was used. The carboxylation of *d*-biotin was followed spectrally (Himes et al., 1963). Protein was determined after deoxycholate/trichloroacetic acid precipitation as described by Bensadoun and Weinstein (1976). Radioactivity was determined by liquid scintillation counting in toluene–Triton X-100 (2:1) containing 2a70 (6 g/L; Research Products Int.). Confirmation of the [^{3}H]biotin prosthetic group was determined by proteinase K (Merck) digestion and paper chromatography as described by Tanabe et al. (1975); the biotin content of MCase and GCase was determined as described by these authors using acid hydrolysis of the proteins and bioassay with *Saccharomyces cerevisiae* (ATCC 9896).

Electrophoresis. Analytical gel electrophoresis at pH 7.9 was carried out at 4 °C as described by Hedrick and Smith (1968). Estimation of the molecular weights of MCase and GCase in this system was carried out by measuring their retardation coefficients as described by Gonenne and Lebowitz (1975), using the following standard proteins (M_R values from Felgenhauer, 1974): apoferritin (473 000), catalase (241 000), conalbumin (86 000), and ovalbumin (43 500). The [^{3}H]-biotin-containing enzymes were detected by slicing the gels into 1-mm slices and then dissolving and counting the slices as described previously (Fall et al., 1975). Enzymatic activity was detected by slicing unstained gels into 2-mm slices and placing each slice into a test tube containing 0.10 mL of 100 mM Tris-HCl, pH 8, 10 mM $MgCl_2$, and 0.5 mg/mL BSA. ATP, $NaH^{14}CO_3$, and appropriate CoA ester substrate were added in the concentrations indicated for the enzymatic [^{14}C]-CO_2-fixation assay of MCase and GCase described above. After incubation for 30 min at 30 °C, the reactions were terminated by the addition of 0.01 mL of 3 N HCl. The contents of each tube was transferred to a scintillation vial which was then heated to dryness at 80 °C. The radioactivity in each vial (both [^{3}H]biotin and [^{14}C]carboxy product) was determined as described in the enzymatic [^{14}C]CO_2-fixation assay above.

Electrophoresis in the presence of $NaDodSO_4$ was carried out pH 7.2 (Weber and Osborn, 1969) or at pH 8.9 (Laemmli, 1970). Samples for $NaDodSO_4$ gels were prepared by (a) deoxycholate/trichloroacetic acid precipitation (Bensadoun and Weinstein, 1976), (b) washing the precipitate with cold acetone (Schwartz and Roeder, 1974), and (c) dissolving the precipitates in 30 μL of 1% $NaDodSO_4$, 1% mercaptoethanol, 20% glycerol, 0.005% bromphenol blue, and 0.01 M Na_3PO_4 at boiling water temperature for 2 min. Protein bands were detected by staining with 0.1% Coomassie blue in 50% ethanol–10% acetic acid, and destained in 10% ethanol–10% acetic acid. For subunit molecular weight determinations, calibration curves were constructed using the following polypeptides (M_R values from Weber and Osborn, 1969): phosphorylase (94 000), bovine serum albumin (68 000), catalase (60 000), ovalbumin (43 000), lactate dehydrogenase (36 000), and carbonic anhydrase (29 000).

Gel Chromatography. The molecular weights of MCase and GCase were estimated by gel permeation chromatography (Andrews, 1970) on a calibrated Sepharose 4B column as previously described (Fall and Vagelos, 1972). The protein standards used were the same as those described above for polyacrylamide gel electrophoresis at pH 7.9. A standard curve was constructed relating log molecular weight to the distribution coefficient, K_{av}, as described by Laurent and Killander (1963).

Enzyme Purification. As previously noted (Hector and Fall, 1976b), MCase from isovalerate-grown cells and GCase from geranoate-grown cells exhibited similar behavior during ammonium sulfate fractionation, and chromatography on DEAE-cellulose, Sepharose 4B, or hydroxylapatite. The purification procedure described below has been used repeatedly for the purification of MCase from isovalerate-grown cells and GCase from geranoate- or citronellate-grown cells. Cells were routinely grown in medium T with the appropriate carbon source, and [^{3}H]biotin to covalently label the biotin prosthetic groups of MCase and GCase as previously described (Hector and Fall, 1976a). All procedures were carried out at 4 °C, and all buffers contained 0.1 mM dithiothreitol.

Step 1. Frozen, washed cells were suspended in 2–3 volumes of buffer K (0.02 M potassium phosphate, pH 7.5), and ruptured by passage through a French pressure cell (20 000 psi). The homogenate was centrifuged at 48 000*g* for 40 min, and the resulting supernatant was used as the source of crude extract.

Step 2. The protein concentration of the crude extract was quickly determined by a microbuiret procedure (Koch and Putnam, 1971) and diluted with buffer K to 10 mg/mL protein, and solid ammonium sulfate was slowly added with stirring to 40% saturation (226 g/L). After sitting for 1 h, the precipitate was collected by centrifugation at 27 000*g* for 20 min, and stored at 4 °C. The supernatant was discarded.

Step 3. The ammonium sulfate precipitate was dissolved in a minimal volume of KG buffer (buffer K containing 20% v/v glycerol) and dialyzed against 100 volumes of KG with three buffer changes for a minimum of 1 h each. The dialyzed fraction was applied to a column of DEAE-cellulose (Whatman DE-52) equilibrated with KG; the bed volume of the column was 1 mL per 20 mg of protein. The column was washed with 1 column volume of KG and then eluted with a linear gradient of KG vs. KG containing 0.3 M KCl using a total gradient volume 10 times the column volume. Typical elution profiles of MCase and GCase have been previously shown (Hector and Fall, 1976b). Both enzymes eluted from the columns at the same conductivity ($\sim$7.5 mΩ^{-1}). The peak enzyme fractions were pooled and precipitated with ammonium sulfate as described in step 2.

Step 4. The precipitate was dissolved in a minimal volume of KG and applied to a column of Sepharose 4B (2.5 × 80 cm) equilibrated with KG; a maximum of 250 mg of protein in a 5-mL volume was applied. The column was eluted with KG and 3–4-mL fractions were collected. Typical elution profiles for MCase and GCase were shown previously (Hector and Fall, 1976b). This step separates a very active ATPase activity from MCase and GCase and allows the use of a spectral assay (Hector and Fall, 1976a).

Step 5. The peak enzyme fractions from step 4 were applied to a column of DEAE-agarose (1 × 30 cm; Bio-Rad) equilibrated with KG. The enzyme was eluted with a linear gradient as described in step 3; the total volume of the gradient was 200 mL and 3-mL fractions were collected. The active MCase or GCase fractions were pooled and concentrated by ultrafiltration (PM 10 membrane, Amicon) to a protein concentration of 2 mg/mL.

Step 6. The concentrated enzyme fractions from step 5 were applied to a column of hydroxylapatite (0.8 × 24 cm; Bio-Rad) equilibrated with KG. Neither MCase nor GCase was adsorbed on the column under these conditions. The nonadsorbed fractions were pooled, concentrated by ultrafiltration to protein

TABLE I: Purification of MCase and GCase of *P. citronellolis*.

Step	MCase[a] Total protein (mg)	MCase[a] Total units	MCase[a] Spec[b] act.	MCase[a] Yield (%)	GCase[a] Total protein (mg)	GCase[a] Total units	GCase[a] Spec[b] act.	GCase[a] Yield (%)
1. Cell extract	8900	267	0.030	(100)	8148	220	0.027	(100)
2. $(NH_4)_2SO_4$ ppt	1639	295	0.18	110	1727	257	0.15	117
3. DEAE-cellulose	199	189	0.95	71	182	162	0.89	74
4. Sepharose 4B	54	105	1.93	39	59	120	2.02	55
5. DEAE-agarose	13.3	73	5.48	27	19.9	87	4.37	40
6. Hydroxylapatite	3.8	40	10.54	15	6.6	55	8.38	25

[a] Cells were grown in T medium containing either 0.3% isovalerate for MCase or 0.15% citronellate for GCase as previously described (Hector and Fall, 1976a). Cells were harvested at the late log stage; approximately 80 g (wet wt) of cells were used in each purification. [b] Specific activity is defined as the carboxylation of 1 μmol of substrate per min per mg of protein at 30 °C. For steps 1–3, a $[^{14}C]CO_2$ fixation assay was used; for steps 4–6, a spectral assay was used (Hector and Fall, 1976a).

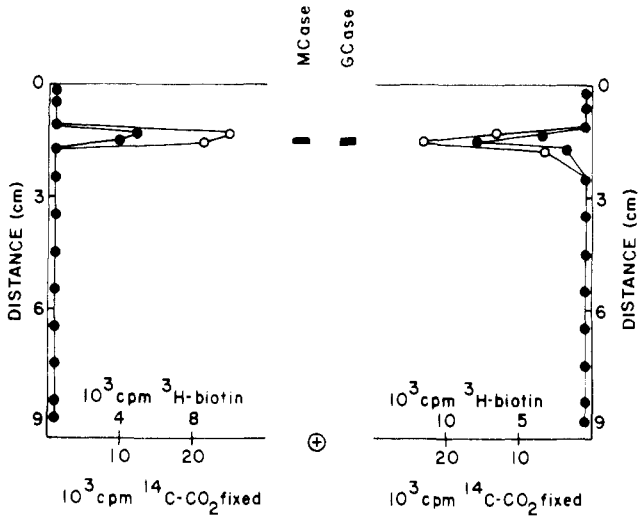

FIGURE 1: Polyacrylamide gel electrophoresis of MCase and GCase at pH 7.9. Acrylamide gels (4.5%) were run as described in the Experimental Procedure. Duplicate gels containing 10 μg of [^{3}H]biotin-labeled MCase or GCase were run, and one was stained for protein and photographed. The other gel was sliced and assayed for enzyme activity and [^{3}H]biotin as described in the text. $[^{14}C]CO_2$ fixed (O—O); [^{3}H]biotin (●—●).

concentration of approximately 1 mg/mL, and stored at −80 °C.

The results of the purification procedure are summarized in Table I. The purified GCase is stable at −80 °C for several months. The purified MCase is less stable, losing approximately 25% activity per month at −80 °C.

Results

Purification of MCase and GCase. Our preliminary attempts to isolate MCase from isovalerate-grown cells and GCase from citronellate-grown cells revealed that the two enzymes exhibited nearly identical purification behavior during ammonium sulfate fractionation, and ion exchange and gel filtration chromatography (Hector and Fall, 1976b). The isolation procedure described under Experimental Procedure was developed for the purification of MCase; addition of glycerol to the buffers in the latter steps was necessary for stabilization of the enzyme. This isolation procedure proved to be equally suitable for the purification of GCase. These procedures resulted in 300–350-fold purification of the two enzymes with 15–25% recoveries of activity (Table I). Both purified preparations contained essentially homogeneous enzymes as described below.

Both enzymes were isolated from cultures grown in the presence of [^{3}H]biotin to label their biotin prosthetic groups. In order to verify that the radioactivity in the purified MCase and GCase preparations was due to covalently bound biotin, each ^{3}H-labeled enzyme was digested for 48 h with an excess of the nonspecific protease, proteinase K, and chromatographed as described by Tanabe et al. (1975). This resulted in one major peak of radioactivity (R_f 0.4) which was indistinguishable from authentic biocytin (ε-*N*-*d*-biotinyl-L-lysine), which constitutes the prosthetic group–amino acid linkage in other biotin–enzymes (Moss and Lane, 1971).

Gel Electrophoresis of the Purified Enzymes. Polyacrylamide gel electrophoresis of the two purified enzymes under nondenaturing conditions at pH 7.9 further revealed the similarity in their size and charge. The results are shown in Figure 1. [^{3}H]Biotin-labeled MCase and GCase each showed a single, identically migrating, protein band. When duplicate gels were sliced and assayed for MCase or GCase, and [^{3}H]biotin, there was coincidence of the protein bands, enzyme activity and the [^{3}H]biotin prosthetic group as shown in Figure 1. This clearly established that the protein band seen in each case is MCase or GCase, respectively. The molecular weight determination of the enzymes from such gels is described below. Only faint traces of other protein bands could be detected on the gels, suggesting that both enzyme preparations are nearly homogeneous.

Polyacrylamide gel electrophoresis of the two purified enzymes in the presence of $NaDodSO_4$ resulted in the detection of two subunits in both enzymes (Figure 2). These subunits are designated the A and B subunits,[2] based on results described below. When a mixture of MCase and GCase was electrophoresced on the same gel using the pH 7.2 gel system of Weber and Osborn (1969), the A subunits showed a slight but reproducible separation, whereas the B subunits migrated identically. Estimation of the molecular weights of these subunits from a calibration curve gave values of 63 000 and 73 000 for the A_M and B_M subunits of MCase, respectively, and 63 000 and 75 000 for A_G and B_G subunits of GCase, respectively.

Electrophoresis of MCase and GCase in the higher resolution $NaDodSO_4$ gel system (pH 8.9) of Laemmli (1970) is

[2] Using the nomenclature of Schiele et al. (1975), A subunit = biotin-free subunit, and B subunit = biotin-containing subunit; the subscript denotes whether the subunit is from MCase (e.g., A_M and B_M) or GCase (e.g., A_G and B_G).

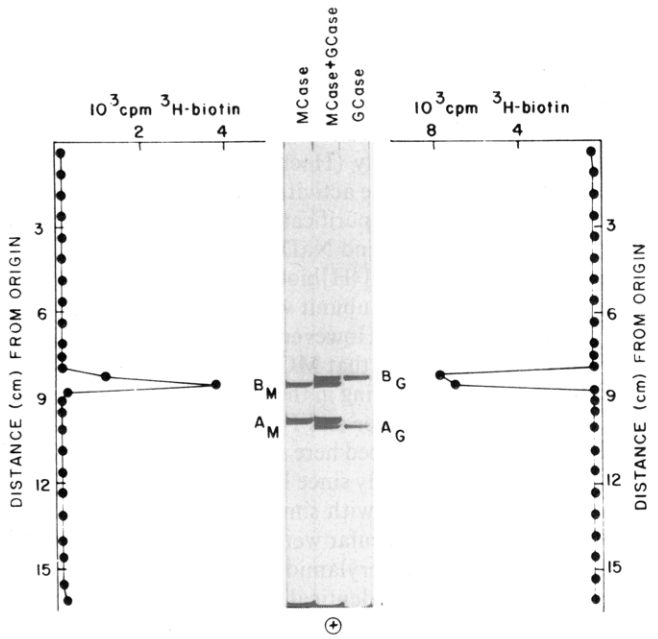

FIGURE 2: $NaDodSO_4$–polyacrylamide gel electrophoresis at pH 8.9 of MCase and GCase. Six percent acrylamide gels (5 × 170 mm) containing 0.1% $NaDodSO_4$ were run as described in the Experimental Procedure. Gels containing 10 μg of [^{3}H]MCase or [^{3}H]GCase, or 10 μg of both enzymes, were run, stained, and photographed. After photography the gels containing MCase or GCase were sliced, digested, and counted (Hector and Fall, 1976a) to locate the [^{3}H]biotin-containing subunit. The designation of the subunits, A_M, A_G, B_M, and B_G is explained in footnote 2.

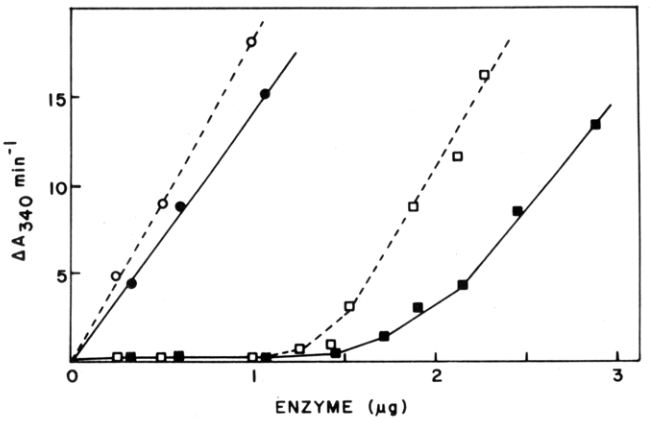

FIGURE 3: Avidin inhibition of MCase and GCase. Increasing amounts of highly purified MCase or GCase were incubated with 1 μg of avidin (13 units/mg) in 0.02 M Tris-Cl, pH 8.0, at 4 °C in 20 μL total volume; MCase (□- -□); GCase (■—■). After 30 min, aliquots were removed for spectral assay. The control incubations contained 10 μg of *d*-biotin mixed with the avidin before addition of the enzymes; MCase (○- -○); GCase (●—●).

shown in Figure 2. Use of this system revealed that the A and B subunits of the two enzymes are not identical; this is clearly seen on a gel containing both MCase and GCase (Figure 2, center gel) where four protein bands are detected. It is not known if the separation of the B subunits in this gel system (and not on pH 7.2 gels) is due to differences in molecular weight or binding of $NaDodSO_4$. It has been noted that discontinuous, alkaline $NaDodSO_4$ gel systems show more anomalous behavior in relation to mobility vs. molecular weight measurements (Neville, 1971; Frank and Rodbard, 1975). Molecular weight determinations from a calibration curve showed more variability and lower values than with the pH 7.2 system. Average values were A_M, 62 000; B_M, 68 000; A_G, 60 000; and B_G, 70 000.

In order to determine which subunit of each enzyme contained the biotin prosthetic group, $NaDodSO_4$ gels containing the [^{3}H]biotin-labeled enzymes were sliced, digested, and counted. Typical results are shown in Figure 2. For both MCase and GCase, only the higher molecular weight subunits (B subunits) contained the ^{3}H-labeled prosthetic group. In this regard the substructure of these enzymes is analogous to that of *Achromobacter* MCase as studied by Lynen and his colleagues (Schiele et al., 1975), which contains a similar complement of A and B subunits.

Molecular Weight. The molecular weights of MCase and GCase were estimated by two different procedures: from gel filtration (Andrews, 1970) and from a determination of retardation coefficient on polyacrylamide gels (Gonenne and Lebowitz, 1975). Gel filtration of MCase, GCase, and protein standards on a Sepharose 4B column as described under Experimental Procedure allowed construction of a standard curve relating log molecular weight to their elution volume. Within experimental error, MCase and GCase eluted from the column at the identical elution volume (K_{av} = 0.23; see also Hector and Fall, 1976b), corresponding to an approximate molecular weight of 520 000 for each enzyme.

Electrophoresis of MCase, GCase, and protein standards on polyacrylamide gels using the pH 7.9 system of Hedrick and Smith (1968) and varying the gel concentration allowed a determination of their retardation coefficients, K_R. MCase and GCase exhibited identical K_R values of 0.47 ± 0.02 cm^3 g^{-1}, corresponding to a molecular weight value of approximately 580 000.

Both of these analytical measurements assume that the enzymes are globular, and other measurements will be needed to further assess the molecular weights of MCase and GCase. However, the estimated molecular weight values of 520 000–580 000 are reasonably consistent with an A_4B_4 structure for these enzymes, assuming the molecular weight values from $NaDodSO_4$ gels are valid and the biotin determinations are accurate (see below).

Biotin Content. Samples of the purified MCase and GCase, digested with proteinase K and then 3 N sulfuric acid as described under Experimental Procedure, were bioassayed for biotin using a *S. cerevisiae* biotin auxotroph which gives a growth response to biotin, biotin sulfoxide, and biocytin (Ogata, 1970), the major hydrolysis products under these conditions. This assay yielded values (μg of biotin per mg of protein) for MCase of 1.65 μg/mg (average of 1.48 and 1.82 for two preparations) and for GCase of 1.56 μg/mg (average of 1.39 and 1.73 for two preparations). These values correspond to 1 mol of biotin per 148 000 g of protein for MCase, and 1 mol of biotin per 157 000 g of protein for GCase. Assuming an approximate molecular weight of 550 000, these values represent 3.5–3.7 biotin residues per enzyme molecule. Since some biotin or active vitamers derived from biotin are destroyed during acid hydrolysis (Tanabe et al., 1975), these values are probably slightly low. These data are consistent with a proposed tetrameric structure (A_4B_4) for these enzymes, where each B subunit contains one biotin prosthetic group.

Avidin Inhibition. Like other biotin-enzymes MCase and GCase are essentially irreversibly inhibited by avidin (Green, 1975). In order to assess the accessibility of their biotin prosthetic group to the inhibitor the experiment in Figure 3 was performed. Increasing amounts of purified MCase or GCase were incubated with a fixed amount of avidin and then assayed for activity. As a control the enzymes were also incubated with biotin-saturated avidin. Incubation with avidin led to inhibition of both MCase and GCase, although there was a quantitative difference in the degree of inhibition of the two enzymes. As

the amount of enzyme mixed with avidin was increased. MCase activity (i.e., saturation of available avidin) appeared at significantly lower concentrations of enzyme than with GCase. Since the two enzyme preparations contain essentially identical amounts of biotin, these results suggest that the biotin prosthetic group of MCase is less accessible to avidin than that of GCase. Incubation of either enzyme in the presence of avidin saturated with biotin resulted in no apparent inhibition as expected (Green, 1975).

Carboxylation of d-Biotin. Several other acyl-CoA carboxylases have been shown to catalyze a model reaction, the ATP-dependent carboxylation of *d*-biotin (see Moss and Lane, 1971). Assays of the purified MCase and GCase showed that both enzymes catalyze this model reaction, although at significantly different rates. MCase carboxylates *d*-biotin at a very low rate, only 0.5–1% the rate of carboxylation of 3-methylcrotonyl-CoA. On the other hand, different GCase preparations carboxylated *d*-biotin at 3–5% the rate of carboxylation of geranyl-CoA. For comparison the MCase from *Achromobacter* carboxylates *d*-biotin at a much higher rate, 24–30% that of 3-methylcrotonyl-CoA carboxylation (Himes et al., 1963). Like the biotin carboxylase component of *Escherichia coli* acetyl-CoA carboxylase (Alberts and Vagelos, 1972), both MCase and GCase have a very low affinity for *d*-biotin ($K_m > 50$ mM), but unlike the *E. coli* enzyme organic solvents do not stimulate the model reaction catalyzed by these two enzymes (R. Fall, unpublished observations).

Discussion

The biotin-dependent carboxylases are a group of enzymes with similar functions, but which show diverse structural features. Each enzyme in this class catalyzes a similar two-step reaction sequence that involves the interactions of three distinct subsites, including a biotin carboxylation site, a transcarboxylation site, and a site containing the biotin prosthetic group that acts as a "CO_2" carrier between the other two sites. In different biotin-dependent carboxylases, these three subsites have been found to be distributed on separate subunits or fused into one or two polypeptide chains as recently reviewed by Obermayer and Lynen (1976). Before this subsite fusion phenomenon was appreciated and at a time when *E. coli* acetyl-CoA carboxylase had been shown to contain these subsites on different functional subunits (Alberts et al., 1969), it was suggested that different biotin-dependent carboxylases in the same organism might share common structural features at the biotin carboxylase and biotin subsites and differ primarily at the transcarboxylase subsite which confers carboxyl acceptor specificity. Two studies aimed at testing this possibility focused on structural comparisons of acetyl-CoA carboxylase and pyruvate carboxylase (another biotin-dependent carboxylase) isolated either from yeast (Sumper and Riepertinger, 1971) or *P. citronellolis* (Fall et al., 1975). Both studies concluded that there are major structural differences between these two enzymes in both organisms. However, in order to rigorously test structural similarities between biotin-dependent carboxylases which contain subsites fused into multifunctional polypeptide chains, as is the case with *P. citronellolis* pyruvate carboxylase and yeast pyruvate and acetyl-CoA carboxylases, it will be necessary to determine amino acid sequence homology. In addition, the two studies cited above have involved comparisons of only two enzymes, acetyl-CoA carboxylase and pyruvate carboxylase, which represent two different classes of biotin enzymes, acyl-CoA carboxylase and α-keto acid carboxylase, respectively (Moss and Lane, 1971). A more relevant comparison might be between biotin enzymes which catalyze homologous reactions (e.g., MCase vs. GCase). Our discovery that *P. citronellolis* contains this homologous pair of enzymes provided the opportunity for such a structural comparison.

Our initial studies with MCase and GCase which were separately induced in *P. citronellolis* by growth on isovalerate or citronellate, respectively (Hector and Fall, 1976a), suggested that the two enzyme activities might be due to one enzyme. Both showed similar purification behavior and molecular weight (by gel filtration) and $NaDodSO_4$ gel electrophoresis of each partially purified [^{3}H]biotin-labeled enzyme preparation suggested a biotin subunit with a molecular weight of ~68 000 (Hector, 1975). However, more recent experiments conclusively demonstrated that MCase and GCase are similar but distinct enzymes, differing in their substrate specificity and heat denaturability (Hector and Fall, 1976b).

The experiments described here show that the two enzymes are very similar structurally since both can be purified by the same isolation procedure with similar yields, and both have essentially the same molecular weight as measured by gel filtration or "native" polyacrylamide gel electrophoresis. The two enzymes appeared identical in the latter procedure. However, the two enzymes were clearly shown to contain different substructures when subjected to high resolution $NaDodSO_4$ gel electrophoresis, showing that each contains different A and B subunits. These results establish that this pair of similar, homologous acyl-CoA carboxylases from the same organism do not share common subunits. And as recently concluded by Obermayer and Lynen (1976), there is still no evidence to support a common subunit hypothesis for different biotin enzymes.

Obviously these results do not rule out the likely possibility that these two homologous enzymes may share common structural features at their three functional subsites. Such similarities are under investigation by peptide mapping of the A and B subunits. In addition we are attempting to dissociate the enzymes into functional A and B subunits by techniques similar to those described by Schiele et al. (1975) with *Achromobacter* MCase, so that intersubunit hybrids can be constructed and tested for compatibility. It is likely that a rigorous test of structural similarity between these two enzymes will require amino acid sequence analysis.

The molecular weights of the two enzymes were estimated to be in the range 520 000–580 000. If the polypeptide molecular weights from $NaDodSO_4$ gels (pH 7.2) are accurate, and the equal staining intensity of the A and B subunits is indicative of an A:B ratio of 1, a tetrametric structure (A_4B_4) with molecular weight 550 000 is suggested for both MCase and GCase. The biotin contents of the two enzymes is consistent with four biotin moieties per A_4B_4 tetramer, presumably with each B subunit containing one biotin prosthetic group. In terms of subunit structure, as well as ability to catalyze the carboxylation of the *d*-biotin, both MCase and GCase from *P. citronellolis* are very similar to the *Achromobacter* MCase characterized by Lynen and his co-workers (Apitz-Castro et al., 1970; Schiele et al., 1975).

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Ligand Binding Stoichiometries, Subunit Structure, and Slow Transitions in Aminoacyl-tRNA Synthetases†

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ABSTRACT: The binding of various combinations of ^{14}C-labeled amino acid, ^{14}C-labeled ATP, and γ-^{32}P-labeled ATP to representative monomeric and dimeric aminoacyl-tRNA synthetases has been studied by equilibrium dialysis, equilibrium and nonequilibrium gel filtration, nitrocellulose disc filtration, and active site titration. The valyl-tRNA synthetase from *Escherichia coli,* a monomer belonging to the class containing regions of duplicated amino acid sequence, forms 1 mol of bound valyl adenylate rapidly. This is followed by the slow binding ($t_{1/2} \sim 11$ min) of an additional mole of chemically unreacted valine, showing that there is a second binding site. Similar evidence has not been obtained for other monomeric enzymes investigated. Another slow process is found in the reactions of the dimeric tyrosyl-tRNA synthetases from *E. coli* and *Bacillus stearothermophilus.* It is known from previous studies that these bind only 1 mol of tyrosine and form only 1 mol of tyrosyl adenylate rapidly per mol of dimeric enzyme. It is now found that a second mole of tyrosyl adenylate is formed and bound with a half-life of several minutes. Although the physiological importance of these slow processes is not known, they provide information on molecular symmetry which is of importance in interpreting the results of x-ray diffraction studies and also provide evidence for the induced fit model of Koshland.

The aminoacyl-tRNA synthetases are a structurally diverse set of enzymes. They may be divided into three main classes with further subdivisions: monomers with molecular weights ranging from 55 000 to 120 000, although typically 110 000; dimers of the form α_2 of molecular weight 2 × 35 000 to 2 × 85 000; and tetramers of the form $\alpha_2\beta_2$ (Soll and Schimmel, 1974). There is the further complication that the monomeric enzymes of molecular weight 110 000 have extensive regions of duplicated primary structure (Koch et al., 1974; Kula, 1973; Waterson and Konigsberg, 1974; Bruton, 1975). One possibility is that these monomers are functionally similar to the dimers of molecular weight about 2 × 50 000; that is, they are effectively "covalently linked" dimers. We have been conducting a comparative survey of the dimeric and monomeric enzymes to see if this is so and to obtain information about the

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