EVIDENCE FOR DISTINCT 3-METHYLCROTONYL-CoA AND GERANYL-CoA CARBOXYLASES IN *PSEUDOMONAS CITRONELLOLIS*

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SUMMARY: A highly purified preparation containing geranyl-CoA and 3-methylcrotonyl-CoA carboxylase activities has been isolated from *Pseudomonas citronellolis* grown on the monoterpene, geranoic acid. These two activities and a separate 3-methylcrotonyl-CoA carboxylase from isovalerate-grown cells exhibited identical purification behavior, suggesting similar ionic properties and molecular weight for these enzymes. The geranyl-CoA carboxylase preparation carboxylated both geranyl-CoA and 3-methylcrotonyl-CoA at relative rates of 1.0 and 0.25, respectively; while 3-methylcrotonyl-CoA carboxylase from isovalerate-grown cells was inactive towards geranyl-CoA. 3-Methylcrotonyl-CoA carboxylase from isovalerate-grown cells was more sensitive to heat denaturation than the geranyl-CoA and 3-methylcrotonyl-CoA carboxylase activities associated with the geranyl-CoA carboxylase preparation. These results suggest that *P. citronellolis* contains two similar but distinct enzymes, 3-methylcrotonyl-CoA carboxylase and geranyl-CoA carboxylase, the latter having a broader acyl-CoA specifity.

Pseudomonas citronellolis grown on geranoic acid as sole carbon source contains both geranyl-CoA and 3-methylcrotonyl-CoA carboxylase activities (1). These enzymatic reactions involve the biotin-dependent carboxylation at the 3-methyl position of homologous acyl-CoA substrates (2,3):

$$R-\underset{\underset{CH_3}{|}}{C}=CHCOSCoA + HCO_3^- + ATP \overset{Mg^{2+}}{\rightleftharpoons} R-\underset{\underset{CH_2CO_2^-}{|}}{C}=CHCOSCoA + ADP + Pi$$

where R = CH_3 (3-methylcrotonyl-CoA carboxylase)

R = $(CH_3)_2C=CHCH_2CH_2$ (geranyl-CoA carboxylase)

Since we have not been able to separate the two activities by standard purification procedures, it has not been clear whether these two activities are due to a) a single geranyl-CoA carboxy-

lase which can carboxylate both geranyl-CoA and 3-methylcrotonyl-CoA, or b) two similar enzymes, geranyl-CoA and 3-methylcrotonyl-CoA carboxylases, which co-purify. Induction of 3-methylcrotonyl-CoA carboxylase with only a trace of geranyl-CoA carboxylase can be effected by growth of *P. citronellolis* on leucine or isovalerate (1), suggesting that the organism probably contains distinct 3-methylcrotonyl-CoA and geranyl-CoA carboxylases which can be induced separately. However, the proposed catabolic pathway for geranoic acid involves production of a 3-methylcrotonyl-CoA intermediate (4) which might act as inducer for 3-methylcrotonyl-CoA carboxylase; alternatively, this intermediate might be metabolized via carboxylation by geranyl-CoA carboxylase (3). This report presents evidence in favor of similar but distinct 3-methylcrotonyl-CoA and geranyl-CoA carboxylases in *P. citronellolis*, with the latter enzyme capable of carboxylating either acyl-CoA.

MATERIALS AND METHODS

The growth of *P. citronellolis*, preparation of cell extracts, and carboxylase assays will be reported (1). Both 3-methylcrotonyl-CoA carboxylase from isovalerate-grown cells and geranyl-CoA carboxylase from geranoate-grown cells were purified by a procedure to be described in detail elsewhere (R. Fall and M. Hector, manuscript in preparation). The general procedure included fractionation of cell extracts between 0-40% saturation with ammonium sulfate, chromatography on DEAE-cellulose using a 0-0.3 M KCl gradient, and then a sequence of chromatography on Sepharose 4B, hydroxylapatite and Sepharose 4B. These procedures yielded carboxylase preparations that were nearly homogenous as measured by polyacrylamide gel electrophoresis.

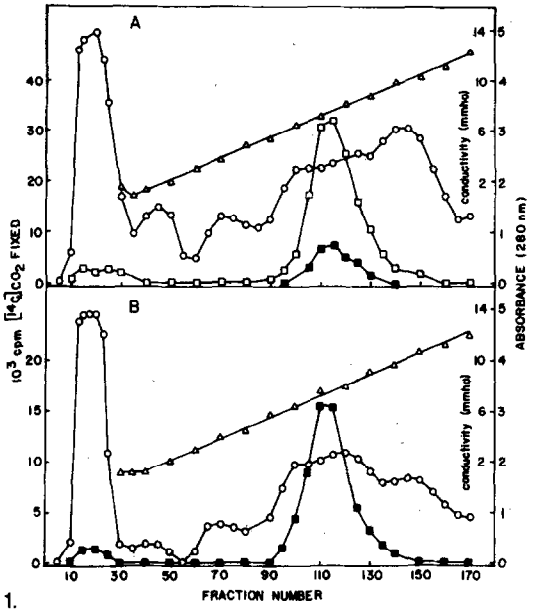
RESULTS AND DISCUSSION

We used a variety of standard purification procedures to attempt to resolve the geranyl-CoA and 3-methylcrotonyl-CoA carboxylase activities present in extracts of geranoate-grown *P. citronellolis*. The two activities exhibited similar

behavior during ammonium sulfate fractionation, and chromatography on DEAE-cellulose, Sepharose 4B or hydroxylapatite. The elution profiles of these activities on DEAE-cellulose are shown in Figure 1. Shown for comparison are the elution profiles for 3-methylcrotonyl-CoA carboxylase from isovalerate-grown cells chromatographed under identical conditions. The geranyl-CoA and 3-methylcrotonyl-CoA carboxylase activities from geranoate-grown cells both eluted from DEAE-cellulose at the same salt concentration (conductivity = 7.5 mmho; Fig. 1A). The ratio of geranyl-CoA to 3-methylcrotonyl-CoA carboxylase activities throughout the peak tubes was 4-5, as measured by a $[^{14}C]CO_2$ fixation assay (1). An identical elution profile from DEAE-cellulose was found for 3-methylcrotonyl-CoA carboxylase from isovalerate-grown cells (Fig. 1B).

Each of the carboxylase peaks eluted from DEAE-cellulose was concentrated by ultrafiltration and chromatographed on Sepharose 4B. As seen in Figure 2 the geranyl-CoA and 3-methylcrotonyl-CoA carboxylase activities from geranoate-grown cells (Fig. 2A) and 3-methylcrotonyl-CoA carboxylase from isovalerate-grown cells (Fig. 2B) emerged at identical elution volumes. This elution volume corresponds to an approximate molecular weight of 520,000 as determined by comparison to the elution positions of proteins of known molecular weight on the same column (R. Fall and M. Hector, manuscript in preparation).

The substrate specificity of each of the higly purified carboxylase preparations was measured using a spectral assay (1,5) as shown in Figure 3. At saturating levels of acyl-CoA substrates the geranyl-CoA carboxylase preparation carboxylated geranyl-CoA and 3-methylcrotonyl-CoA at relative rates of 1.0 and 0.25 respectively (Fig. 3A); this ratio of activities is



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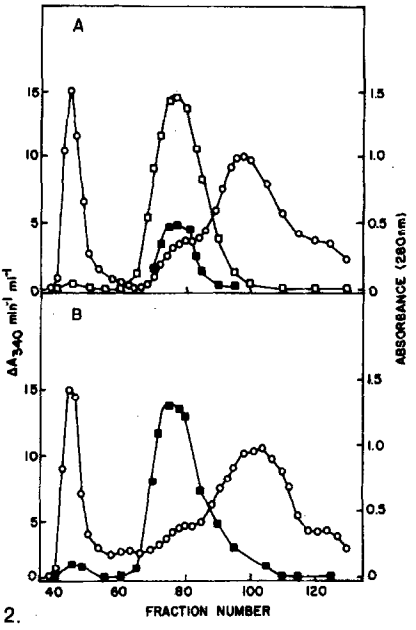

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Figure 1. DEAE-cellulose chromatography of geranyl-CoA and 3-methylcrotonyl-CoA carboxylase preparations. Ammonium sulfate fractions (400-500 mg protein) from either isovalerate or geranoate-grown cells were dialyzed against 0.02 M potassium phosphate, pH 7.5, containing 0.1 mM dithiothreitol and 20% glycerol (buffer A) and applied to a column of DEAE-cellulose (2.5 x 12 cm) equilibrated with buffer A. The columns were eluted with a linear gradient of 350 ml buffer A versus 350 ml buffer A containing 0.3 M KCl; 5-ml fractions were collected. The salt concentration was measured by conductivity (Δ—Δ); protein was detected by absorbance at 280 nm (O—O). Geranyl-CoA carboxylase (□—□) and 3-methylcrotonyl-CoA carboxylase (■—■) were assayed by a [^{14}C]CO_2 fixation assay (1) using 0.01 ml aliquots and 5 min incubations. (A) Chromatography of the carboxylase preparation from geranoate-grown cells. (B) Chromatography of the carboxylase preparation from isovalerate-grown cells.

Figure 2. Sepharose 4B chromatography of geranyl-CoA and 3-methylcrotonyl-CoA carboxylase preparations. The carboxylase peaks obtained from DEAE-cellulose (Fig. 1) were concentrated by ammonium sulfate precipitation, dissolved in 3-ml of buffer A and chromatographed on a column of Sepharose 4B (2.5 x 80 cm) in buffer A; 3-ml fractions were collected. Carboxylase assays were performed optically on 0.01 ml aliquots (1). The symbols are the same as in Fig. 1. (A) Chromatography of the carboxylase preparation from geranoate-grown cells. (B) Chromatography of the carboxylase preparation from isovalerate-grown cells.

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the same as that seen throughout the purification with the [^{14}C]CO_2 fixation assay and noted above. In a previous study with a partially purified geranyl-CoA carboxylase preparation

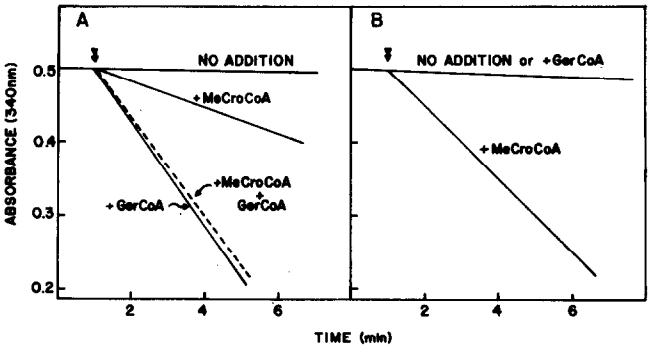

Figure 3. Acyl-CoA specificity of purified geranyl-CoA and 3-methylcrotonyl-CoA carboxylase preparations. The highly purified carboxylase preparations were assayed optically by coupling ADP formation to NADH oxidation (1,5). Reactions were initiated by addition of acyl-CoA(s) (indicated by the arrows). (A) Assay of the geranyl-CoA carboxylase preparation (0.5 µg protein) with no acyl-CoA added or with the indicated acyl-CoA(s) at 0.17 mM. (B) Assay of the 3-methylcrotonyl-CoA carboxylase preparation (0.7 µg protein) as in (A). Ger-CoA = geranyl-CoA; MeCro-CoA = 3-methylcrotonyl-CoA.

Seubert *et al.* (3) observed relative rates of 17.7 and 1.0 with geranyl-CoA and 3-methylcrotonyl-CoA, respectively. The reason for the difference in ratios of the two carboxylase activities between their study and ours is not clear. Possibly the difference is related to the purity of the 3-methylcrotonyl-CoA preparations used; Knappe *et al.* (6) noted that an addition product between coenzyme A and 3-methylcrotonyl-CoA can be formed during the synthesis of the latter. We could not detect this addition product in our preparations of 3-methylcrotonyl-CoA. Under similar assay conditions the 3-methylcrotonyl-CoA carboxylase from isovalerate-grown cells would not carboxylate geranyl-CoA (Fig. 3B).

To test the possibility that the purified geranyl-CoA carboxylase preparation was a mixture of distinct geranyl-CoA and 3-methylcrotonyl-CoA carboxylases which had co-purified, we assayed in the presence of both geranyl-CoA and 3-methyl-

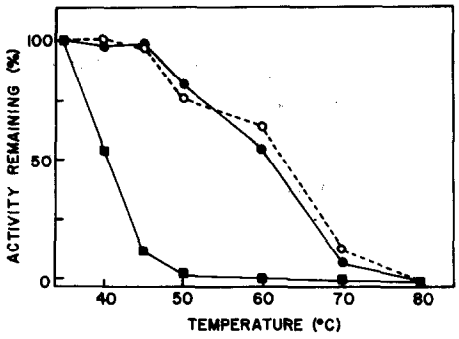

Figure 4. Heat inactivation of geranyl-CoA and 3-methylcrotonyl-CoA carboxylase preparations. Samples of each highly purified carboxylase preparation were incubated in 0.05 M sodium phosphate, pH 7.5, containing 0.5 mM EDTA and 0.1 mM dithiothreitol for one minute at the indicated temperatures and then immediately assayed at 30° as described in the legend of Fig. 3. The activities at 30° are taken as 100%. Geranyl-CoA (O···O) and 3-methylcrotonyl-CoA (●—●) carboxylase activities purified from geranoate-grown cells; 3-methylcrotonyl-CoA carboxylase (■—■) from isovalerate-grown cells.

crotonyl-CoA (Fig. 3A). As seen, the reaction rate was not additive, suggesting that the preparation does not contain two carboxylases. To further test this possibility we subjected the geranyl-CoA carboxylase and 3-methylcrotonyl-CoA carboxylase preparations to heat denaturation. The results are shown in Figure 4. The 3-methylcrotonyl-CoA carboxylase from isovalerate-grown cells was much more susceptible to heat denaturation, losing 50% of its activity during heating at 40 C for one minute. In contrast the two carboxylase activities associated with the geranyl-CoA carboxylase preparation lost 50% activity only after heating one minute at 60 C, and they were denatured in parallel, suggesting they reside on the same protein.

These results strongly suggest that isovalerate-grown *P. citronellolis* contains a distinct 3-methylcrotonyl-CoA carboxylase, and the 3-methylcrotonyl-CoA carboxylase activity present in geranoate-grown cells is due to geranyl-CoA carboxylase alone. Thus it seems likely that the 3-methylcrotonyl-

CoA fragment, which is a postulated intermediate in the geranoate degradative pathway (4), is metabolized via geranyl-CoA carboxylase, and does not induce a distinct 3-methylcrotonyl-CoA carboxylase.

These results are especially interesting in relation to our studies of the structural aspects of the multiple subsites of biotin-dependent acyl-CoA carboxylases (1), since it appears that 3-methylcrotonyl-CoA and geranyl-CoA carboxylases represent a pair of homologous enzymes that can be isolated from the same organism. As recently shown by Lynen and coworkers (7) 3-methylcrotonyl-CoA carboxylase from *Achromobacter* is composed of two dissimilar subunits with molecular weights of 78,000 and 96,000. The larger subunit contains the biotin carboxylase subsite and the biotin prosthetic group, and the smaller subunit probably contains the transcarboxylase subsite. It seems possible that the 3-methylcrotonyl-CoA and geranyl-CoA carboxylases of *P. citronellolis*, which are also each composed of two dissimilar subunits analogous to those of *Achromobacter* 3-methylcrotonyl-CoA carboxylase (R. Fall and M. Hector, manuscript in preparation) share structural features in common, such as the biotin carboxylase and biotin subsites, and differ only at the transcarboxylase subsite which confers acyl-CoA specificity. This possibility is under investigation.

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REFERENCES

1. Hector, M. L., and Fall, R. R., Biochemistry, in press.
2. Alberts, A. W., and Vagelos, P. R. (1972) The Enzymes, Vol. 6, pp. 37-82, Academic Press, New York.
3. Seubert, W., Fass, E., and Remberger, U. (1963) Biochem. Z. 338, 265-275.

4. Seubert, W., and Fass, E. (1964) Biochem. Z. 341, 35-44.
5. Himes, R. H., Young, D. L., Ringelmann, E., and Lynen, F. (1963) Biochem. Z. 337, 48-61.
6. Knappe, J., Schlegel, H-G., and Lynen, F. (1961) Biochem. Z. 335, 101-122.
7. Schiele, U., Niedermeier, R., Stürzer, M., and Lynen, F. (1975) Eur. J. Biochem. 60, 259-266.