*LIVER ACETYL COENZYME A CARBOXYLASE: ACTIVATION OF MODEL PARTIAL REACTIONS BY TRICARBOXYLIC ACIDS**

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Previous reports from this laboratory[1, 2] have demonstrated that isocitrate (or citrate) activation of liver acetyl coenzyme A (CoA) carboxylase (E.C. 6.4.1.2) is accompanied by polymerization of the protomeric form (molecular weight, 410,000). The active polymeric form was found to have a unique filamentous structure and a molecular weight of about 4–5 million. A conformational change in the vicinity of the active site was indicated[3, 4] by the decreased accessibility of the biotinyl prosthetic group to the specific biotin-binding protein, avidin, in the presence of activator (isocitrate or citrate). In addition to the over-all carboxylation and decarboxylation reactions (reaction (3)), both partial reactions (reactions (1) and (2)) appeared to be affected as evidenced by a "citrate effect" on the ATP-P_i^{32} and malonyl-CoA–C^{14}-acetyl-CoA exchange reactions.[2, 4-6]

$$\text{Enz} + \text{ATP} + \text{HCO}_3^- \overset{Mg^{2+}}{\rightleftharpoons} \text{Enz-CO}_2^- + \text{ADP} + P_i \qquad (1)$$

$$\text{Enz-CO}_2^- + \text{acetyl-CoA} \rightleftharpoons \text{Enz} + \text{malonyl-CoA} \qquad (2)$$

$$\text{Over-all: ATP} + \text{HCO}_3^- + \text{acetyl-CoA} \overset{Mg^{2+}}{\rightleftharpoons} \text{ADP} + P_i + \text{malonyl-CoA} \qquad (3)$$

Nevertheless, attempts to study the kinetics of enzyme-CO_2^- formation and the subsequent carboxyl transfer from enzyme-CO_2^- to acetyl-CoA have been unsuccessful because of the rapidity of these reactions. This communication demonstrates that liver acetyl-CoA carboxylase catalyzes the carboxylation of free (+)-biotin and that the carboxylated enzyme (enzyme-CO_2^-) transfers its carboxyl group to acetyl pantetheine to form malonyl pantetheine. These reactions, which serve as enzymatic models for the forward half reactions (1) and (2), respectively, can be followed kinetically and are both activated by citrate and isocitrate. The bearing of this evidence on the mechanism of activation by tricarboxylic acids is discussed.

Experimental Procedure.—Acetyl-CoA carboxylase was prepared in homogeneous form from chicken liver and carboxylase assays were conducted as previously described.[1, 6] The preparation of enzyme-$C^{14}O_2^-$ was carried out as reported by Ryder *et al.*[4] We are indebted to Drs. O. Wis and W. E. Scott, Hoffman-La Roche, Inc. (Basle, Switzerland and Nutley, New Jersey, respectively) for supplying (+)- and (−)-biotin, (+)-norbiotin, (+)-homobiotin, d,l-O-heterobiotin, and d,l-dethiobiotin; and to Merck Sharpe and Dohme Research Laboratories for a sample of biocytin (ε-N-(+)-biotinyl-L-lysine). 2-Imidazolidone (ethyleneurea) obtained from Aldrich Chemical Co., Inc. was recrystallized from absolute ethanol. S-acetyl-D-pantetheine was prepared according to the procedure described by Hegre and Lane.[7]

Carboxylation of free biotin: The rate of carboxylation of free biotin was determined

by a modification of the method of Lynen *et al.*[8] The complete reaction mixture contained the following components (in μmoles per ml, unless specified): tris(hydroxymethyl)-aminomethane (Tris) (Cl^-) buffer, pH 7.5, 60; (+)-biotin, 20; adenosine 5′-triphosphate (ATP), 2; $MgCl_2$, 8; $KHC^{14}O_3$ (specific radioactivity, 5–7 $\times$ 10^6 cpm per μmole), 10; potassium citrate, 10; dithiothreitol, 2; bovine serum albumin, 0.6 mg per ml; and acetyl-CoA carboxylase (specific activity, 8–9 units per mg of refractometrically determined protein), about 1 mg per ml. After incubation at 37° for periods up to 8 min, the reaction was terminated by the rapid transferal of 0.1-ml aliquots to 0.8 ml of water (0–2°) containing 1 drop of n-octanol and by the bubbling of CO_2 through the solution for 40 min at 0–2° to remove the excess $HC^{14}O_3^-$. No significant loss of carboxybiotin occurred during the gassing procedure. The gassed solution was made alkaline and residual C^{14}-activity (1′-N′-carboxybiotin) was determined with a liquid scintillation spectrometer. Since the half life of carboxybiotin under the reaction conditions (37°) was found to be 28 min, losses of carboxybiotin by decarboxylation during the 8-min incubation period were considered negligible. Appropriate corrections were made for radioactivity present as enzyme-$C^{14}O_2^-$.

Results.—Effect of tricarboxylic acid activator on the carboxylation of free (+)-biotin: Although it was commonly believed[9] that the carboxylation of free biotin was a unique property of the microbial β-methylcrotonyl-CoA carboxylase studied extensively in Lynen's laboratory,[8, 10, 11] this view is no longer tenable. As illustrated in Figure 1, liver acetyl-CoA carboxylase catalyzed the carboxyla-

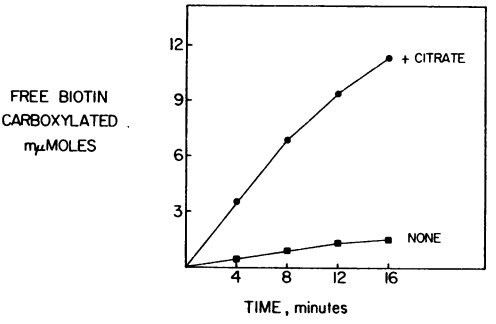

FIG. 1.—Kinetics of carboxylation of free biotin. The carboxylation of free (+)-biotin was determined as described in *Experimental Procedure.* The total quantity of free biotin carboxylated in 1.0 ml of reaction mixture containing 0.8 mg of acetyl-CoA carboxylase is indicated on the vertical axis. Potassium citrate was added at a level of 10 mM as indicated.

tion of free (+)-biotin. Moreover, citrate (or isocitrate) activated the reaction seven- to eightfold. Since this reaction serves as a model for the initial step (reaction (1)) in the over-all carboxylation of acetyl-CoA, it is evident that activation of this step occurs. Linear carboxylation rates were usually obtained for up to eight minutes at 37°. The carboxylation product was identified as 1′-N-carboxybiotin by methylation with diazomethane and by comparison of the chromatographic behavior of the methylated product with authentic 1′-N-methoxycarbonyl biotin methyl ester as described by Knappe *et al.*[11]

An investigation of the effect of citrate on the Michaelis constant for biotin revealed a significantly higher K_m (40 mM) in the presence of citrate than in its absence (K_m = 8 mM). Free biotin concentrations greater than 20 mM are inhibitory. It is not possible, therefore, to achieve saturation with the citrate-activated carboxylase. The maximal velocity of free biotin carboxylation in the presence of citrate is 4.5 mμmoles per minute per mg of protein (refractometrically determined), which is about 1/2000 the rate of acetyl-CoA carboxylation.

The K_m for citrate of 5 mM in the former reaction is in good agreement with those observed for the over-all carboxylation[2] and ATP-P_i^{32} exchange[6] reactions.

The carboxylation reaction exhibits a high degree of structural and stereochemical specificity with respect to biotin. The effects of certain alterations of the biotin molecule on carboxylation rate are shown in Table 1. Whereas covalent binding serves to firmly anchor the prosthetic group to the apoprotein through its 14 Å side-chain,[12] secondary binding sites must also be involved for proper orientation of the bicyclic biotin ring relative to bound HCO_3^-, with which it reacts. The existence of secondary enzyme sites for the biotinyl ring and its side-chain amide group are indicated by the reactivity of biotin and biocytin and by the reduced activity or lack of reaction with biotin analogues.

TABLE 1. *The specificity of the carboxylation of free biotin.*

Derivative	Concentration (mM)	Carboxylation rate* relative to (+)-biotin (%)
(+)-Biotin	20	100
(−)-Biotin	20	0
Biocytin	20	122
(+)-Homobiotin	20	31
(+)-Norbiotin	20	28
d,l-O-Heterobiotin	40	27
d,l-Dethiobiotin	40	0
2-Imidazolidone (ethyleneurea)	20–100	0

* Carboxylation rates were determined as described in *Experimental Procedure;* biotin derivatives were substituted for (+)-biotin in the reaction mixture.

Biocytin (ε-N-(+)-biotinyl-L-lysine), which has a side-chain amide group in the same position as the biotinyl prosthetic group of the enzyme, is a somewhat better substrate than free biotin. On the other hand, the biotin side-chain homologues, norbiotin (C_4) and homobiotin (C_6), are much less reactive than biotin (C_5). This indicates that the distance from the carboxyl group to the thiophane ring is critical, and in the case of biotin optimal. The inactivity of dethiobiotin and 2-imidazolidone (ethyleneurea), as well as the reduction in activity caused by the substitution of O for S in the thiophane ring, as with oxybiotin (O-heterobiotin), emphasizes the importance of the ring structure in binding. It may further be seen from Table 1 that a change in configuration about the 2-position (the point of juncture of the side chain with the thiophane ring), i.e., from (+)- to (−)-biotin, completely abolishes activity.

The above observations suggest that a specific enzyme site hydrogen-bonds the side-chain amide of the prosthetic group or, alternatively, the carboxyl or amide of free biotin or its analogues. The high degree of specificity, as well as the high K_m for (+)-biotin, supports the view that free biotin and certain of its derivatives bind and are carboxylated at the site normally occupied by the bicyclic ring of the biotinyl prosthetic group.

Effect of tricarboxylic acid activator on carboxyl transfer from enzyme-$C^{14}O_2^-$ *to S-acetyl-D-pantetheine:* Earlier investigations[4] demonstrated that isocitrate (or citrate) markedly activated the decarboxylation of enzyme-CO_2^-. This indicated that activation by tricarboxylic acids increased the reactivity of the N-

carboxy group of the carboxybiotinyl enzyme and might have been expected to enhance carboxyl transfer to acetyl-CoA. Stoichiometric carboxyl transfer to acetyl-CoA was found to be too rapid to follow kinetically by conventional methods under a wide variety of reaction conditions (at pH's from 6.5 to 8.0 and at temperatures from 2° to 37°, and with various buffering media and ionic strengths). Therefore, a valid test of the effect of the tricarboxylic acid activator on this reaction was not possible. On the other hand, as illustrated in Figure 2, carboxyl transfer from enzyme-CO_2^- to acetyl pantetheine, a poorer substrate, can be followed kinetically. The transfer reaction exhibits a dependence upon acetyl pantetheine concentration and an almost absolute requirement for isocitrate; similar results have been obtained with citrate. The trans-

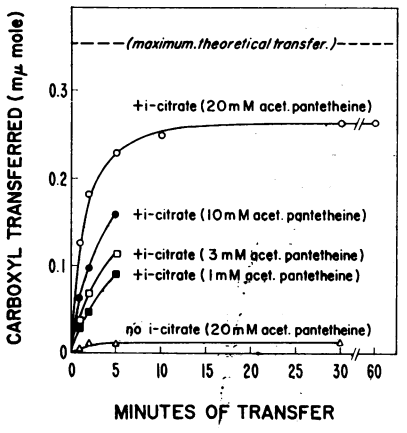

FIG. 2.—Kinetics of carboxyl transfer from enzyme-CO_2^- to acetyl pantetheine to form malonyl pantetheine. Enzyme-$C^{14}O_2^-$ (0.35 mμmole; specific radioactivity, 2.5 × 10^4 cpm per mμmole), prepared as described earlier,[4] was incubated at 25° in a reaction mixture (1 ml total volume and final pH,7.5) that contained 60 μmoles of Tris (Cl^-) buffer, 3 μmoles of glutathione (GSH), 0.1 μmole of ethylenediaminetetraacetate (EDTA), and the levels of S-acetyl-D-pantetheine and potassium D,L-isocitrate indicated. Aliquots were withdrawn at the times indicated and acidified with HCl, and acid-stable radioactivity (C^{14}-malonyl pantetheine) was determined as describe ealier.[1]

fer product was identified as S-malonyl pantetheine by methods described earlier.[13, 14] The K_m for acetyl pantetheine in the over-all carboxylation reaction (ATP-dependent C^{14}-bicarbonate fixation assay) is 6.3 × 10^{-3} M compared to a K_m of about 10^{-5} M for acetyl-CoA,[2] whereas the maximal velocities are approximately 0.1 and 9.2 μmoles per minute per mg of protein, respectively. Activation of the transfer reaction does not appear to alter the affinity of the carboxylase for acceptor. It is evident from Figure 2 that the K_m for acetyl pantetheine in the transfer reaction, i.e., 5–10 mM, is similar to that (K_m = 6.3 mM) for the over-all carboxylation reaction. Like the K_m for acetyl-CoA,[2] the K_m for acetyl pantetheine in the over-all carboxylation reaction is not affected by isocitrate. The affinity of the enzyme for acetyl-CoA as evidenced by binding experiments is also unaffected by the presence of citrate.[15] These results are consistent with the view that activation by isocitrate (or citrate) increases the reactivity of the N-carboxy group and hence its transfer to appropriate acceptors, including acetyl pantetheine.

Discussion.—Earlier investigations[1, 2, 4] have shown that citrate and isocitrate, which activate liver acetyl-CoA carboxylase, promote the concomitant polymerization of the carboxylase protomer as well as a conformational change in the vicinity of the biotinyl prosthetic group. The primary kinetic effect of the activators appears to be on the maximal velocities of the reactions catalyzed by the

enzyme.[4, 6] The present communication shows that in addition to the known reactions of liver acetyl-CoA carboxylase,[1-5] the enzyme also catalyzes the carboxylation of free biotin, and in its carboxylated form (enzyme-CO_2^-), transfers its carboxyl group to acetyl pantetheine. These reactions serve as enzymatic models for the two half reactions (reactions (1) and (2)) in the carboxylation of acetyl-CoA and offer the advantage of being sufficiently slow to be followed kinetically. Like the over-all process, both model reactions are markedly activated by tricarboxylic acid activators. It is clear from these results and other facts[4] concerning the reaction that both partial reactions are activated by citrate. Since both half reactions involve a common biotinyl prosthetic group, it is conceivable that the V_{max} effect of the activator may be directed at this functional group. Such an effect could result if substrate sites or specific activating groups were brought into closer proximity to the prosthetic group. Furthermore, since the protomer is composed of four subunits,[15] it may be that the activation process involves a reorientation of these subunits rather than a simple redistribution of groups on a single polypeptide chain. The validity of this concept is currently under investigation.

It has already been established[4] that the reactivity of the N-carboxy group of carboxybiotinyl enzyme (enzyme-CO_2^-) is greatly enhanced by isocitrate. Further insight into the effect of citrate on the biotinyl prosthetic group was gained in experiments on the carboxylation of free biotin. The high degree of specificity for (+)-biotin or closely related derivatives in this reaction indicates that binding and carboxylation of the free species occur at the same specific site normally occupied by the bicyclic ring of the biotinyl prosthetic group. In addition to a covalent attachment to the enzyme (in amide linkage to lysyl ϵ-amino groups as in other biotin enzymes), the prosthetic group would appear, from the evidence, to require secondary binding sites; these include a site for the biotin bicyclic ring and a hydrogen-bonding site for the side-chain amide group. The high K_m for free biotin reflects the difficulty with which the prosthetic group is displaced from these secondary sites while remaining covalently attached. The substantial length, 14 Å, of the side chain[12] could readily accommodate this maneuver. The fact that the K_m for free biotin is increased by citrate suggests that the prosthetic group under these conditions may be shielded more by neighboring groups and, therefore, is displaced only by higher concentrations of free biotin. This interpretation is supported by the observation[4] that the biotinyl prosthetic group is less accessible to complexing by avidin in the citrate-activated enzyme.

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[1] Gregolin, C., E. Ryder, A. K. Kleinschmidt, R. C. Warner, and M. D. Lane, these PROCEEDINGS, **56**, 148 (1966).

[2] Gregolin, C., E. Ryder, R. C. Warner, A. K. Kleinschmidt, and M. D. Lane, these PROCEEDINGS, **56**, 1751 (1966).

[3] Lane, M. D., C. Gregolin, E. Ryder, A. K. Kleinschmidt, and R. C. Warner, in Symposium on Regulation of Lipid Metabolism, *Abstracts*, American Chemical Society Meeting, Chicago, August 1967, p. 261 C.

[4] Ryder, E., C. Gregolin, H. C. Chang, and M. D. Lane, these PROCEEDINGS, **57**, 1455 (1967).

[5] Matsuhashi, M. S., S. Matsuhashi, and F. Lynen, *Biochem. Z.*, **340**, 263 (1964).

[6] Gregolin, C., E. Ryder, and M. D. Lane, *J. Biol. Chem.*, in press.

[7] Hegre, C. S., and M. D. Lane, *Biochim. Biophys. Acta*, **128**, 172 (1966).

[8] Lynen, F., J. Knappe, E. Lorch, J. Jutting, E. Ringelmann, and J. P. Lachance, *Biochem. Z.*, **335**, 123 (1961).

[9] Ochoa, S., and Y. Kaziro, in *Comprehensive Biochemistry*, ed. M. Florkin and E. H. Stotz (Amsterdam: Elsevier Publishing Co., 1965), vol. 16, p. 244.

[10] Knappe, J., H. G. Schlegel, and F. Lynen, *Biochem. Z.*, **335**, 101 (1961).

[11] Knappe, J., E. Ringelmann, and F. Lynen, *Biochem. Z.*, **335**, 168 (1961).

[12] Although the biotinyl prosthetic group is covalently linked in amide linkage to lysyl ϵ-amino groups in biotin enzymes, it is evident that its functional bicyclic ring must be bound at a secondary site on the enzyme for the carboxylation to take place. Measurements made with Corey-Pauling models (Koltun connectors) of ϵ-N-(+)-biotinyl-L-lysine reveal that the maximum distance from the α-carbon to the juncture of the side chain with the thiophane ring is about 14 Å.

[13] Halenz, D. R., and M. D. Lane, *J. Biol. Chem.*, **235**, 878 (1960).

[14] Lane, M. D., D. R. Halenz, D. P. Kosow, and C. S. Hegre, *J. Biol. Chem.*, **235**, 3082 (1960).

[15] Gregolin, C., E. Ryder, R. C. Warner, A. K. Kleinschmidt, H. C. Chang, and M. D. Lane, *J. Biol. Chem.*, in press.