**Communication**

**Biotin Carboxylations—Concerted or Not Concerted? That Is the Question!***

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**SUMMARY**

When $\beta$-fluoropropionyl coenzyme A is used as substrate, propionyl-CoA carboxylase catalyzes the formation of ADP and the elimination of fluoride ion. No F$^-$ release occurs in the absence of ATP or in the presence of avidin. ADP formation occurs as rapidly as in the presence of propionyl-CoA, but the rate of F$^-$ release is 6 times that of ADP formation. The rate of F$^-$ release is indicative of the minimal rate of abstraction of the proton, and the rate of ADP formation is equivalent to the rate of formation of biotin-CoA. The results, therefore, show that hydrogen abstraction can occur without concomitant CO$_2$ transfer from biotin-CoA to the substrate. Therefore, the concerted mechanism which has been proposed for this, and other biotin enzymes, is not applicable when propionyl-CoA carboxylase acts on $\beta$-fluoropropionyl-CoA. We believe the concerted mechanism is also not involved in the carboxylation of the normal substrate, propionyl-CoA.

Propionyl-CoA carboxylase (EC 6.4.1.3) from pig heart catalyzes the following reactions:

$$E$$-biotin + HCO$_3^-$ + ATP $\xrightarrow{Mg^{2+}}$ $E$-biotin-CO$_2$ + ADP + P$_i$

$$E$$-biotin-CO$_2$ + propionyl-CoA $\xrightarrow{\text{E-biotin + MeMal-CoA}}$

This reaction, like other biotin-dependent carboxylations, involves the abstraction of the $\alpha$ proton and its replacement by CO$_2$. Rébay and Lysen (1) showed that the reaction catalyzed by propionyl-CoA carboxylase, proceeds with retention of configuration, and proposed that proton abstraction and carboxylation occur in a concerted process (Fig. 1). An important feature of the concerted mechanism is that the carbonyl group of biotin serves as proton acceptor in one direction and as proton donor in the other. The dissociation of the bond between the carboxyl group and the biotin nitrogen increases the negative charge density on the biotin carbonyl group, and this enables the carbonyl group to function as proton acceptor. Perhaps the strongest support for the concerted mechanism comes from the work of Rose et al. (2) with transcarboxylase (Equation 2). When the enzyme acts

$$\text{CH}_3\text{CO-COO}^- + \text{CH}_3\text{CH} \xrightarrow{\text{COOH} + \text{CH}_3\text{CO-COO}^-} \text{CH}_3\text{CO} - \text{CH}_3\text{CO-COO}^-$$

$\text{COSeA} + \text{CH}_3\text{CH}_2\text{COSeA}$

on $[^1\text{H}]$pyruvate and methylmalonyl-CoA, a small but significant number of counts are transferred to the C-2 position of the resulting propionyl-CoA. Some tritium transfer from propionyl-CoA to pyruvate also occurs in the reverse direction. The authors feel that these results can be best interpreted by a concerted mechanism, where biotin serves as proton donor and acceptor. However, they also propose an alternative, although in their view less attractive, possibility which does not require a concerted mechanism. The concerted mechanism has been proposed for this, and other enzymes, by several investigators (3–5).

The alternative to the concerted mechanism is a stepwise process involving $\alpha$ proton abstraction followed by carboxylation. Evidence for such proton abstraction would be provided if exchange between substrate protons and solvent protons could be detected in the absence of carboxylation, or if it could be shown that such an exchange reaction is more rapid than the carboxylation. No evidence of this kind can be obtained for propionyl-CoA carboxylase (3) or for other biotin-dependent enzymes (5). It is, of course, possible that the enzyme catalyzes proton abstraction in the absence of carboxylation or prior to carboxylation, but the abstracted proton does not exchange with solvent protons.

We decided to examine the action of propionyl-CoA carboxylase on $\beta$-fluoropropionyl-CoA since this compound appeared ideally suited to determine whether proton abstraction occurs in the absence of carboxylation. The properties of $\beta$-fluoropropionyl-CoA which make it particularly suitable are: (a) fluoride is relatively small, and therefore substitution of fluorine for hydrogen will not cause steric problems. (b) When a carbanion is generated $\beta$ to carbon bearing a fluorine atom, fluorine is readily eliminated. Therefore, F$^-$ elimination will be indicative of carbanion formation. This lability of the C–F bond was pointed out by Santi (6) and is the basis for a number of irreversible enzyme inactivators (7–9).

$\beta$-Fluoropropionyl-CoA was incubated in the presence of HCO$_3^-$, ATP, and Mg$^{2+}$. The experimental details and results are shown in Fig. 2. When $\beta$-fluoropropionyl-CoA, ATP, and HCO$_3^-$ are added to the enzyme, ADP is formed and F$^-$ is released. No F$^-$ is released in the absence of ATP. No F$^-$ or ADP formation occurs in the presence of avidin. The rate of ADP formation is the same as that observed in the presence of propionyl-CoA. However, the initial rate of F$^-$ release is 6 times as fast as ADP formation. No conversion of H$^+$CO$_3^-$ to an acid-stable carboxyl group could be detected, i.e. no evidence for the formation of fluoromethylmalonyl-CoA was

* We have shown that $\beta$-fluorothioesters eliminate F$^-$ at pH 7.0. For example, $t_{1/2}$ for F$^-$ elimination for the N-acetylcysteamine derivative of $\beta$-fluoropropionic acid is 73 min (PD 1.2; 1 M K+, PO$_4^-$); for the $\beta$-Cl compound, $t_{1/2}$ is >400 h under the same conditions.
obtained. The rate of F⁻ release is presumably an indication of the rate of abstraction of the substrate α proton, and the rate of ADP formation indicates the rate of biotin-CO₂ formation. Therefore, the rate of proton abstraction is at least 6 times faster than the carboxylation of biotin. Hence, abstraction of the α-hydrogen of fluoropropionyl-CoA can occur without concomitant carboxyl transfer from biotin-CO₂ to the substrate.

Scheme 1 shows the reaction sequence which we believe is consistent with our results. Reaction of the substrates with E-boottin leads to the formation Complex I. A base on the enzyme then abstracts the substrate α proton to form the enzyme-bound carbanion II. The carbanion eliminates F⁻ to form Complex III, which no longer contains a CO₂ acceptor. The elimination is more rapid than the transfer of CO₂ from biotin to the carbanion. Complex III releases acrylyl-CoA and is converted to E-boottin-CO₂. E-boottin-CO₂ has a sufficiently long life to catalyze HF elimination from 5 molecules of fluoropropionyl-CoA through I, II, and III. E-boottin-CO₂ must eventually decompose and it can do so through several possible routes (indicated by dotted arrows in Scheme 1). It could decarboxylate to regenerate E-boottin. In view of the relatively long half-life of enzyme-boottin-CO₂ complexes (5), we consider this unlikely and prefer one of the following: (a) Complex III decarboxylates. It is possible, although at this point we have no supporting evidence, that in the presence of acrylyl-CoA, the rate of decarboxylation of E-boottin-CO₂ is increased. (b) The fluoropropionyl-CoA carbanion (Complex II) is carboxylated in one out of six turnovers to produce fluoromethylmalonyl-CoA, which decarboxylates during work up. Again, we have no experimental data concerning the stability of fluoromethylmalonyl-CoA.

The results reported here are considered strong evidence that propionyl-CoA carboxylase can abstract an α proton from fluoropropionyl-CoA without concomitant CO₂ transfer from biotin-CO₂; therefore, there must be a group at the active site which functions as proton acceptor. The possibility that the F⁻ is removed by a displacement reaction rather than via formation of a carbanion through α proton removal is considered highly unlikely. We believe that nonconcerted proton abstraction also occurs with "normal" substrates. Failure to detect enzyme-catalyzed proton exchange with propionyl-CoA is then due to the ability of enzyme to prevent exchange with solvent protons. The lack of exchange also suggests that the base at the active site of the enzyme is a monoprotic base.

**Synthesis of Fluoropropionyl-CoA** — Fluoropropionyl chloride was prepared by adding 1 eq of SOCl₂ (3.33 g, 0.028 mol) to fluoropropionic acid (2.5 g, 0.027 mol) in an ice bath,
FIG. 2. Reaction of fluoropropionyl-CoA with propionyl-CoA carboxylase. The assay solution contained 0.1 mM Tris, pH 7.8, 4 mM MgCl₂, 2 mM reduced glutathione, 1 mM ATP, 1 mM phosphoenolpyruvate, 0.1 mM KCl, 50 mM NaHCO₃, 50 μg pyruvate kinase (specific activity, 160 μmol/min/mg), 0.38 mM NADH, 50 μM lactate dehydrogenase (specific activity, 560 μmol/min/mg), and 0.175 mM fluoropropionyl-CoA. Final volume 0.75 ml, temperature 24°C. The reaction was started by the addition of 0.0025 mg of enzyme (purified by the procedure of Kaziro (10), specific activity, 2.4 pmol/min/mg). ADP formation; 0, complete reaction mixture with avidin (0.6 mg/ml), 0.12 μmol/30") into a dry ice/acetone receiver. Recovery of the compound. The acid chloride is carefully distilled (20 to 30°C) in a dry ice/acetone receiver. Great care should be taken not to allow the solution to become too basic. After completion of the reaction, 10 min, the pH of the solution is adjusted to 3.5 to 4.0 with concentrated HCl. The solution is then extracted with a portion (3 x 5 ml) of ether to remove the excess fluoropropionic acid. The pH is then readjusted to 4.8 and the crude mixture is purified by paper chromatography.

Purification by Paper—Whatman No. 3MM paper (8 x 24 inches) is spotted with 4 to 6 mg of the crude CoA reaction mixture. The paper is developed in butanol:acetone:acetic acid:1 M NH₄Ac (9:3:2:6) over a period of 16 to 20 h. The spots are visualized by uv light or by taking a small strip of the paper and testing for a positive nitroprusside test with base (a red spot). The fluoropropionyl-CoA analog R₈ = 0.23, is easily separated from slower moving, nitroprusside negative, contaminants.

β-Fluoropropionyl-CoA was characterized by its nmr spectrum. Assignments were made based on the spectrum of β-fluoropropionyl-N-acetylcysteamine and the spectra of CoA analogs already published (11). We have included the spectral data for the β-fluoropropionyl-N-acetylcysteamine derivative for comparison with the corresponding data for the CoA analog. The remainder of the CoA assignments are identical with those reported (11).

β-Fluoropropionyl-N-acetylcysteamine—δ = 1.86 (3H, singlet); δ = 2.99 (2H, doublet of triplets, JCHF = 28.5 Hz, JCHCH = 8 Hz); δ = 3.04 (2H, broad doublet); δ = 3.30 (broad triplet, J = 6.7 Hz); δ = 4.68 (2H, doublet of triplets, JCHF = 46.5 Hz, JCHCH = 6 Hz).

β-Fluoropropionyl-CoA —Ref. δ = 0.80 (6H, doublet, J = 11.7 Hz); δ = 2.97 (2H, broad doublet); δ = 2.96 (doublet of triplets, JCHF = 29.1 Hz, JCHCH = 6 Hz); δ = 3.30 (broad triplet, J = 7 Hz); δ = 4.67 (doublet of triplets, JCHF = 46.7 Hz, JCHCH = 6 Hz).

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