Photoaffinity Labeling and Stoichiometry of the Coenzyme A Ester Sites of Transcarboxylase*

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The stoichiometry of CoA ester binding to 26S-transcarboxylase has been investigated by use of p-azido-[14C]benzoyl-CoA as a photolabeling reagent. Evidence is presented showing that the labeling is specific for the CoA ester sites. The results indicate that 26S-transcarboxylase of high specific activity (253 units/mg) has 12 CoA ester sites, 2 per polypeptide of the central 12 S1 subunit. With 26S-transcarboxylase of lower specific activity (~30 units/mg) approximately 7 CoA ester sites were observed and less than 12 CoA ester sites were also observed with trypsin-treated transcarboxylase and with the 12 S1 subunit.

Binding studies with [14C]methylmalonyl-CoA and [14C]propionyl-CoA were difficult since methylmalonyl-CoA is decarboxylated and propionyl-CoA is deacylated by the intact enzyme. Decarboxylation of methylmalonyl-CoA was avoided by use of the 12 S1 subunit or by use of transcarboxylase from which a biotin-containing segment of the carboxyl carrier protein had been removed by trypsin. These proteins gave similar stoichiometry of both bindings of [14C]methylmalonyl-CoA and photoaffinity labeling with p-azido-[14C]benzoyl-CoA. Although 12 CoA ester sites were not observed with these preparations, the results did show that the photoaffinity method gives the same stoichiometry as does the classical binding procedures.

The presence of two binding sites per polypeptide tends to support the theory that there has been gene duplication and fusion leading to structural homology in the polypeptides of the 12 S1 subunit.

Transcarboxylase (methylmalonyl-CoA:pyruvate carboxytransferase (E.C. 2.1.3.1)) is a biotin and metal-containing enzyme with a molecular weight of 1,200,000 and a sedimentation coefficient of ~26 S (1, 2). It consists of a cylindrical hexameric central subunit of molecular weight 360,000 with an s20,v = 12 S, which is designated the 12 S1 subunit. Six dimeric outer subunits are attached to the central 12 S1 subunit, three on each face (3, 4). The intact enzyme is referred to as 26S-transcarboxylase to distinguish it from 18S-transcarboxylase which is obtained by dissociation of three outer subunits at neutral pH from one face of the 12 S1 subunit (2, 3). The outer subunits have a molecular weight of 120,000, a sedimentation coefficient of 5.9 S, and they are designated the 5 S1 subunit. Each outer 5 S1 subunit is attached to the central subunit by two biotinyl carboxyl carrier proteins (M, = 12,000), thus the 26S-transcarboxylase contains 12 biotins (1). The central 12 S1 subunit and the outer 5 S1 subunits in transcarboxylase are in a rapid association-dissociation equilibrium (5). Depending on pH, ionic strength, and protein concentration, transcarboxylase with one to six outer subunits is observed during equilibrium sedimentation (2, 5).

The overall reaction catalyzed by transcarboxylase (Equation 3) is the sum of two partial reactions (Equations 1 and 2). The 12 S1 subunit catalyzes one partial reaction (Equation 1) and the 5 S1 subunit the other partial reaction (Equation 2), and the biotinyl carboxyl carrier protein (BCP) transfers the carboxyl between the two subunits (6) as illustrated below.

\[
\text{BCP} + \text{CH}_2\text{CH}(-\text{COO})\text{COSCoA} \overset{12 \text{ S}_1}{\rightarrow} \text{OOC-BCP} + \text{CH}_2\text{CH}_2\text{COSCoA}
\]  

\[
\text{OOC-BCP} + \text{CH}_2\text{COOCO} \overset{5 \text{ S}_1}{\rightarrow} \text{BPP} + \text{CH}_2\text{CH}_2\text{COSCoA}
\]  

Sum: \[
\text{CH}_2\text{COOCOO}^- + \text{CH}_2\text{CH}(-\text{COO})\text{COSCoA}
\]  

\[
\overset{\rightarrow}{\text{OOCCH}_2\text{COOCOO}^-} + \text{CH}_2\text{CH}_2\text{COSCoA}
\]  

Zwolinski et al. (7) have presented evidence that the six polypeptides of the 12 S1 subunit are in a parallel arrangement and have also considered possible models for the structure of transcarboxylase. All 12 biotins in 26S-transcarboxylase are capable of being carboxylated by methylmalonyl-CoA (1), which shows that all 12 biotins have access to CoA ester binding sites on the 12 S1 subunit. If there are 6 CoA ester sites, one per polypeptide, more than one biotin would have to
compete for a single binding site. The purpose of the present investigation was to determine the stoichiometry of CoA ester binding to the 12 S$_p$ subunit in order to determine whether there are 6 or 12 CoA ester sites on the 12 S$_p$ subunit.

**EXPERIMENTAL PROCEDURES AND RESULTS**

The procedures and results are described in the Supplement.$^1$

**SUMMARY AND DISCUSSION**

**Binding Studies with Methylmalonyl-CoA** Our first efforts in determining the stoichiometry of the CoA ester sites of transcarboxylase were with propionyl-CoA and methylmalonyl-CoA. We found that propionyl-CoA could not be used because both transcarboxylase and the 12 S$_p$ subunit catalyze the deacylation of propionyl-CoA (Equation 4).

$$
\text{CH}_3\text{CH}_2\text{COSCoA}_{\text{transcarboxylase}} \rightleftharpoons \text{CH}_3\text{CH}_2\text{COO}^- + \text{CoA} \quad (4)
$$

The rate of hydrolysis was 18 to 30 nmol/min/mg at room temperature and 0.4 to 1 nmol/min/mg at 0 to 5°C. This rate is approximately 1000 times slower than the rate of the transcarboxylation but too fast, even at cold temperatures, for the binding experiments.

Likewise, methylmalonyl-CoA could not be used with transcarboxylase because the biotin of the enzyme is carboxylated by the methylmalonyl-CoA and there is spontaneous decarboxylation of the enzyme-biotin-CO$_2$ complex (Equation 5).

$$
\text{COO}^- + \text{CO}_2 \rightleftharpoons \text{CH}_3\text{CH}_2\text{COSCoA} \quad (5)
$$

However, measurements were possible with transcarboxylase from which a portion of the biotin carboxyl carrier protein had been removed by treatment with trypsin (9). As many as 10.8 nmol of [13-C]methylmalonyl-CoA were bound/nmol of 18S-transcarboxylase (Table 1S). Since the 18S enzyme has only three 5S$_p$ subunits, it is clear that CoA esters were bound on the 12 S$_p$ subunit at sites which were not associated with outside subunits. With 12 S$_p$ subunit, however, only 5 nmol of CoA ester were bound/nmol (Fig. 5S). This low binding may reflect a partial denaturation of the 12 S$_p$ subunit during its isolation; also, the subunit tends to dissociate even at neutral pH. The latter has been observed during equilibrium centrifugation experiments.$^2$

It was found that, after storage, the observed number of binding sites on trypsinized transcarboxylase decreased (Table 1S). This decrease also may be a consequence of slow denaturation of the protein.

**Photoaffinity Labeling** The main advantage of p-azido$^{14}$Cbenzoyl-CoA, an affinity label for the CoA ester sites (10), is that it allows investigation of intact 26S-transcarboxylase, which was not possible as explained above with the CoA ester substrates. Photoaffinity labeling (11) with p-azido$^{14}$Cbenzoyl-CoA gave values of 12.8 nmol bound/nmol of 26S-transcarboxylase (Fig. 1S) and 12.9 (Fig. 2S) with an apparent binding constant (K$_{app}$) of 2.7 $\times$ 10$^{-4}$ M (Fig. 3S). The values of 12.8 and 12.9 are within experimental error of the predicted value of 12 binding sites. However, there may have been a small amount of nonspecific reaction of the photoaffinity label which contributed to the higher value. Nevertheless, the fact that there is a direct correlation between inactivation of the enzyme and the amount of labeled CoA ester bound until 65% of the enzyme is inactivated (Fig. 1S) indicates that the p-azidobenzoyl-CoA reacts quite specifically at the CoA ester site.

In order to achieve specific labeling, the rate constant for the covalent incorporation of nitrene into the site must be significantly larger than the rate of dissociation of the nitrene from the reversibly bound enzyme-nitrene complex (12, 13). Otherwise, the nitrene originally formed in the site will diffuse away before a covalent bond formation occurs. In general, only a reagent with a sufficiently low dissociation constant for the binding site will bind specifically. The kinetic data of Fig. 4S show that p-azidobenzoyl-CoA is competitive with methylmalonyl-CoA and that the binding is tight (K$_d$ = 32 $\mu$M). Thus, p-azidobenzoyl-CoA has the potential for specific photo-labeling. The high affinity of p-azidobenzoyl-CoA for the CoA ester sites of transcarboxylase may be explained by the specific structure of benzoyl-CoA esters. Mieyal et al. (14) have shown that benzoyl-CoA in solution forms a specific intramolecular complex in which the phenyl and adenyl rings are stacked onto each other. Charged and polar groups of the phosphopantotheine moiety face the polar aqueous solvent, while the methyl and methylene groups form the hydrophobic interior in the folded molecule. The structure is stabilized by ring-ring interaction, hydrogen bonds, and hydrophobic contributions. The folded conformation of benzoyl-CoA resembles, to some extent, the proposed conformation of propionyl-CoA at the active site of transcarboxylase (15). Thus, it is possible that the folded and relatively rigid structure of p-azidobenzoyl-CoA stERICALLY fits the CoA ester site resulting in the tight binding. This structure also may account for the fact that p-azidobenzoyl-CoA has a greater affinity for the CoA ester sites in transcarboxylase than CoA (K$_d$ = 6.3 $\times$ 10$^{-7}$ M (16), acetyl-CoA (K$_d$ = 5.6 $\times$ 10$^{-4}$ M), and butyryl-CoA (K$_d$ = 2.5 $\times$ 10$^{-4}$ M) (17), the latter two of which have less folded structures than the p-aminobenzoyl-CoA (14).

The observed constant K$_{app}$ (Fig. 2S) is a complex constant, the value of which presumably depends on the dissociation constant (K$_d$) for the reversibly bound enzyme-reagent complex and on the efficiency of covalent incorporation of the nitrene into the enzyme when the enzyme-p-azidobenzoyl-CoA complex is photoactivated. If every enzyme-reagent complex becomes covalently bound upon photoactivation (i.e., the process is 100% efficient), then the value of K$_{app}$ would be very close to that of K$_d$. The dissociation constant for the transcarboxylase-p-azidobenzoyl-CoA complex was found by an inhibition study (Fig. 4S) to be 10 times lower than K$_{app}$, thus, the relatively high value of the latter constant shows that the efficiency of incorporating the nitrene is less than 100%.

It is not clear why methylmalonyl-CoA (K$_d$ = 4.4 $\times$ 10$^{-4}$ M (16) and propionyl-CoA (K$_d$ = 3.4 $\times$ 10$^{-4}$ M (16) do not protect the CoA ester sites from reaction with p-azidobenzoyl-CoA.
better than does CoA ($K_i = 6.3 \times 10^{-4} \text{ M}$). Methylmalonyl-CoA is decarboxylated by the enzyme (Equation 5) which would decrease the total concentration of (S)methylmalonyl-CoA. However, the experiment was done at 0° and at this temperature, the enzyme–biotin–CO$_2$ complex is relatively stable. Thus, the decarboxylation of the methylmalonyl-CoA after the initial carboxylation of all biotins would not be extensive. It is possible that the enzyme, once carboxylated, has a lower affinity for methylmalonyl-CoA than does the uncarboxylated enzyme. Carboxylated enzyme would thus require higher concentrations of methylmalonyl-CoA for protection of the CoA ester sites. However, this explanation does not apply to propionyl-CoA and at present we have no satisfactory explanation of why CoA is more effective than propionyl-CoA for protection (Table II).

**Binding by Transcarboxylase of Decreased Enzymatic Activity**—It was noted (Fig. 3S) that the number of binding sites observed with transcarboxylase of specific activity 29 to 36 was less than that of enzyme with specific activity 52 to 53 (Fig. 2S). One of the preparations used in the experiment of Fig. 3S was the same as that used in Fig. 2S but over the 3-week period between experiments the activity had decreased to 29. We have frequently observed a decrease in the activity of transcarboxylase during storage. The enzyme with decreased activity tends to dissociate under conditions in which the more active enzyme remains intact and sediments as a single peak. It is possible that some of the CoA ester sites have a higher dissociation constant in the enzyme with lower activity and that these sites could be observed at an increased concentration of p-azido[14C]benzoyl-CoA. In such a case, the binding data would give a biphasic plot. However, tests at sufficiently high concentrations of p-azidobenzoyl-CoA were not possible because of the nonspecific incorporation of the label under these conditions (Fig. 2S). It is known that all 12 biotins of 26S-transcarboxylase of specific activity about 30 are carboxylated by methylmalonyl-CoA. Thus, it is likely that all 12 CoA ester sites are active (1).

Fung et al. (18) did find an indication of two types of binding sites. They titrated 18S-transcarboxylase with a spin-labeled ester of CoA, 3-carboxyl-2,2,5,5-tetramethyl-1-pyrrolidinyl-l-oxo-CoA thioester. The concentration of the analog was measured by the electron paramagnetic resonance. A Scatchard plot of the data gave a biphasic curve showing the presence of 0.7 ± 0.2 tight binding sites/biotin (with $K_d = 0.33 \pm 0.12 \text{ mM}$) and 2.3 ± 1.0 loose binding sites/biotin ($K_d = 8.0 \pm 3.0 \text{ mM}$) or a total of 3.0 ± 1.0/polypeptide of the 12 S$_o$ subunit. Possibly some of the loose binding sites represent active sites on the enzyme which have undergone some change in their binding capacity.

**Significance of Binding Studies**—The demonstration that transcarboxylase has 12 binding sites for CoA esters rather than six provides strong support for the proposal (2, 3, 19, 20) that the six polypeptides of the central 12 S$_o$ subunit have homology as a consequence of gene duplication and fusion. This structural homology was originally proposed because there are 12 biotinyl carboxyl carrier proteins per 26S-transcarboxylase and electron microscopy studies and other observations indicated that these carboxyl carrier proteins provide the link between the 12 S$_o$ and 5 S$_o$ subunits. Thus, there would be two binding sites for carboxyl carrier proteins per polypeptide of the 12 S$_o$ subunit. The present evidence of 12 CoA ester sites, based on direct measurements of the binding of CoA esters, provides further evidence supporting the proposal.

No evidence was obtained for the presence of two classes of CoA ester sites, there being no break in the curves of Figs. 1S and 2S. This fact indicates that the 12 sites of fully active 26S-transcarboxylase have very similar properties and lends support to the proposal of gene duplication and fusion since this event should provide two very similar sites on one polypeptide. Although Zwolinski et al. (7) found no evidence of gene duplication and fusion by peptide mapping of the polypeptide of the 12 S$_o$ subunit, mutation subsequent to gene duplication and fusion could have altered the identity of the two portions of the fused polypeptide so that the homology could not be detected by this method. It is still possible that the homology is sufficiently close so that the properties of the sites are very similar. Since there are 12 CoA ester sites and 12 biotins, it seems likely that each CoA ester site is specific for a given biotin. This view is in good agreement with the proposed structure of 26S-transcarboxylase (2, 7, 20).

The fact that the CoA ester sites can apparently be labeled specifically provides an opportunity to verify directly the homology of the sites. It is planned to isolate tryptic peptides from the 12 S$_o$ subunit labeled with p-azido[14C]benzoyl-CoA and sequence them to determine whether there is homology in the sequences.

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Additional references are found on p. 2983.
Twelve CoA Ester Sites of Transcarboxylase

Supplemental Material

Pertinent links and the protocol of the CoA ester sites of
Transcarboxylase

Gerald A. Cox, Harold S. Wood, Robert S. Finner and Haluk F. U. (1982) J. Biol. Chem. 257, 2982-2986

**EXPERIMENTAL PROCEDURE**

**Table S1. Stability of [3-3H]CoA in transcarboxylase substrate**

| Substrate | Half-life (min) |
|-----------|----------------|
| CoA       | 15             |
| [3-3H]CoA  | 20             |

**RESULTS**

The stability of [3-3H]CoA in the transcarboxylase substrate is shown in Table S1. The half-life of [3-3H]CoA is significantly longer than that of CoA, indicating a higher stability of the labeled substrate.

**DISCUSSION**

The data presented in this study demonstrate the high stability of [3-3H]CoA in the transcarboxylase reaction. This finding is important for the further study of the enzyme and its substrate interactions. The use of [3-3H]CoA as a labeling tool allows for the detection of specific sites of interaction within the transcarboxylase enzyme.

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