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Chemical Modification of Tryptophan Residues in *Escherichia coli* Succinyl-CoA Synthetase. Effect on Structure and Enzyme Activity†

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ABSTRACT: Succinyl-CoA synthetase of *Escherichia coli* is an $\alpha_2\beta_2$ protein containing active sites at the interfaces between α- and β-subunits. The α-subunit contains a histidine residue that is phosphorylated during the reaction. The β-subunit binds coenzyme A and probably succinate [see Nishimura, J. S. (1986) *Adv. Enzymol. Relat. Areas Mol. Biol. 58*, 141–172]. Chemical modification studies have been conducted in order to more clearly define functions of each subunit. Tryptophan residues of the enzyme were modified by treatment with *N*-bromosuccinimide at pH 7. There was a linear relationship between loss of enzyme activity and tryptophan modified. At one tryptophan residue modified per β-subunit, 100% of the enzyme activity was lost. In this enzyme sample, one methionine residue in each α- and β-subunit was oxidized to methionine sulfoxide, although loss of enzyme activity could not be related in a linear manner to the formation of this residue. Subunits were prepared from enzyme that was inactivated 50% by *N*-bromosuccinimide with 0.5 tryptophan modified per β-subunit but with insignificant modification of methionine residues in either subunit. Small decreases in the tyrosine and histidine content were observed in the α-subunit but not in the β-subunit. In this case, modified β-subunit when mixed with unmodified α-subunit gave a population of molecules that was 50% as active as the refolded, unmodified control but was only slightly changed with respect to phosphorylation capacity and unchanged with respect to rate of phosphorylation. Relatively slight changes were observed in the phosphorylation capacity of the α-subunit both alone or when refolded with β-subunit. The results suggest that modification of a tryptophan residue in the β-subunit causes loss of catalytic activity but does not interfere with its ability to refold with the α-subunit to establish a conformation of the latter that is similar to that in the native enzyme. Failure of substrates and the CoA analogue desulfo-CoA to protect the enzyme against inactivation by *N*-bromosuccinimide would appear to indicate that the modified tryptophan residue is not at the active site.

Succinyl-CoA synthetase catalyzes the reaction

$$\text{NTP} + \text{CoA} + \text{succinate} \xrightleftharpoons{M^{2+}} \text{NDP} + P_i + \text{succinyl-CoA} \quad (1)$$

where NTP[1] and NDP are purine ribonucleoside triphosphate and diphosphate, respectively, and M^{2+} is a divalent metal ion. Covalent steps in catalysis by the enzyme are described in eq 2a–c (Nishimura & Grinnell, 1972; Bridger, 1974).

$$\text{NTP} + \text{enzyme} \rightleftarrows \text{enzyme-P} + \text{NDP} \quad (2a)$$

$$\text{enzyme-P} + \text{succinate} \rightleftarrows \text{enzyme·succinyl-P} \quad (2b)$$

$$\text{enzyme·succinyl-P} + \text{CoA} \rightleftarrows \text{enzyme} + \text{succinyl-CoA} + P_i \quad (2c)$$

Succinyl-CoA synthetase from *Escherichia coli* contains an $a_2\beta_2$ subunit structure. In the phosphorylation step (see eq 2a), the α-subunit is phosphorylated (Bridger, 1971). The β-subunit apparently contains at least part of the CoA binding site (Collier & Nishimura, 1978) and, perhaps, the succinate

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[1] Abbreviations: NTP, purine ribonucleoside triphosphate; NDP, purine ribonucleoside diphosphate; P_i, inorganic phosphate; ATP, adenosine 5′-triphosphate; NBS, *N*-bromosuccinimide; SCS, succinyl-CoA synthetase; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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binding site (Benson et al., 1969; Pearson & Bridger, 1975b). Pearson and Bridger observed that, while the α-subunit is capable of autophosphorylation by ATP, the presence of the β-subunit is required for optimal phosphorylation rates. Recent studies reported from this laboratory indicate that all six tryptophan residues are contained in the two β-subunits (Prasad et al., 1983a). In addition, the fluorescence properties of at least one of these tryptophans in each β-subunit is affected significantly by the binding of CoA and ATP to the enzyme.

In this study, we present evidence that oxidation of a tryptophan residue in the β-subunit is sufficient to abolish enzyme activity but that phosphorylation of the α-subunit by ATP can still occur.

Experimental Procedures

Materials. The sodium salts of ATP and *N*-bromosuccinimide were obtained from Sigma. [^{32}P]ATP was purchased from New England Nuclear.

Enzyme. Succinyl-CoA synthetase was isolated from *E. coli* as described previously (Bowman & Nishimura, 1975). The enzyme was assayed by the method of Kaufman et al. (1953), as modified by Grinnell and Nishimura (1969). The method depends on the continuous conversion of succinyl-CoA in the presence of hydroxylamine to succinyl hydroxamate, which is detected as its ferric complex (Lipmann & Tuttle, 1945).

Fractionation of Subunits. The α- and β-subunits of succinyl-CoA synthetase were isolated essentially as described by Pearson and Bridger (1975a). This method involves separation of the subunits on a Sephadex G-150 column that is equilibrated with an acidic urea solution. Elution of the larger β-subunit was preceded by a peak that appeared to be aggregated material. This peak was larger in the case of the *N*-bromosuccinimide-modified enzyme. However, separation of α- and β-subunits from each other was still satisfactory. Measurement of the subunit protein was made by the method of Lowry et al. (1951).

Radioactive Measurements. Samples were dissolved in Hydrofluor scintillation fluid (National Diagnostics) and counted in a Tracor Analytical Model 6892 scintillation counter.

Amino Acid Analysis. Protein samples were hydrolyzed in 6 N HCl at 110 °C for 24 h. Amino acid analysis was carried out in a Durrum D-500 amino acid analyzer by Dr. Katherine Wall.

Quantitation of Methionine Sulfoxides in Protein. The method described by Neumann (1967) was used.

Quantitation of Tryptophan Residues. The method of Sasaki et al. (1975) was used predominantly in this study. The method of Spande and Witkop (1967a) was used in one experiment (see Figure 2). Values from the application of the two methods were quite comparable.

Fluorescence Measurements. Fluorescence emission spectra were measured in a Perkin-Elmer MPF 44a spectrofluorometer.

Results

We have shown previously that substrates, such as ATP-Mg^{2+} and CoA, have a significant effect on the tryptophan fluorescence of *E. coli* succinyl-CoA synthetase (Prasad et al., 1983a). These observations were consistent with the idea that at least one of the three tryptophan residues is close to or at the active site of the enzyme. It was therefore of interest to determine what consequences, if any, tryptophan modification might have on catalytic and other properties of the enzyme.

The sensitivity of succinyl-CoA synthetase to *N*-bromosuccinimide at pH 7 is demonstrated by the results described in Figure 1. Nearly 100% inactivation was achieved at a ratio of modifying agent to enzyme of 20. At ratios of 30 and 50, complete inactivation was observed (data not shown). Such sensitivity to the reagent was not surprising, since the enzyme is known to be inactivated in oxidizing environments, at least partly through disulfide bond and cysteic acid residue formation (Nishimura et al., 1976). Incubation of the modified enzyme with 0.1 M dithiothreitol at pH 7–8 showed only a slight tendency to reverse the inactivation. Unfolding and refolding of modified enzyme in the presence of 0.1 M dithiothreitol (Prasad et al., 1983b) also had no significant effect. The likelihood that disulfide bond formation was a major factor in this case would appear to be ruled out. Therefore, it seemed likely that inactivation of the enzyme by *N*-bromosuccinimide involved an irreversible modification of the enzyme. The possible formation of cysteic acid residues alluded to above was ruled out by the absence of cysteic acid in acid hydrolysates of the modified enzyme.

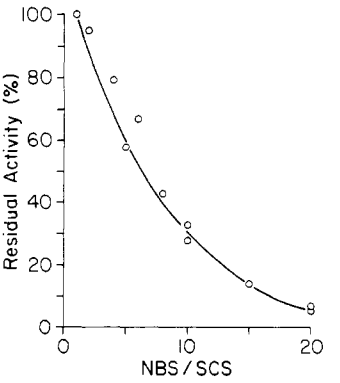

FIGURE 1: Inactivation of succinyl-CoA synthetase by *N*-bromosuccinimide. The reaction mixtures contained 50 mM sodium glycerophosphate (pH 7), 7.1 μM succinyl-CoA synthetase [specific activity 1030 μmol/(30 min·mg)], and *N*-bromosuccinimide at ratios of reagent to enzyme of from 1 to 20. Incubation was carried out for 5 min at 23 °C. Suitable samples were assayed for residual enzymatic activity. NBS, *N*-bromosuccinimide; SCS, succinyl-CoA synthetase.

Table I: Modification of Tyrosine and Histidine Residues by *N*-Bromosuccinimide[a]

	tyrosine		histidine	
subunit	observed	expected[b]	observed	expected[b]
unmodified α	5.41	6	6.32	6
modified α	4.70		5.49	
unmodified β	6.52	7	5.70	5
modified β	6.16		6.09	

[a] Succinyl-CoA synthetase was modified at a ratio of *N*-bromosuccinimide to enzyme of 20, as described in Figure 1. Modified subunits were separated from each other by the method of Pearson and Bridger (1975a). Amino acid analysis of the subunits was conducted as described under Experimental Procedures. The results given represent the arithmetic average of the data from five experiments. [b] Buck et al. (1985).

The possible oxidation of tyrosine and histidine residues by *N*-bromosuccinimide (Schmir & Cohen, 1961) was considered. Analysis of separated α- and β-subunits for tyrosine and histidine following oxidation of the enzyme at a ratio of *N*-bromosuccinimide to enzyme of 20 gave the results shown in Table I. There was a decrease of almost one residue each of these amino acids in the modified α-subunit. The same decrease in tyrosine was observed at a ratio of 5 (data not shown). On the other hand, changes in the tyrosine and histidine content of the β-subunit were not significant.

Methionine residues of proteins are known to be oxidized by reagents such as *N*-bromosuccinimide (Schechter et al.,

Table II: Formation of Methionine Sulfoxides during Modification of *E. coli* Succinyl-CoA Synthetase by *N*-Bromosuccinimide[a]

ratio of NBS to SCS	% inactivation	subunit	MetSO found (mol/mol of subunit)
5	43	α	<0.1
		β	<0.1
10	75	α	0.2
		β	<0.1
20	88	α	1.2
		β	1.0

[a] Enzyme was modified as described in Figure 1. Modified subunits were separated from each other by the method of Pearson and Bridger (1975a). Analysis for methionine sulfoxides was performed according to the method of Neumann (1967).

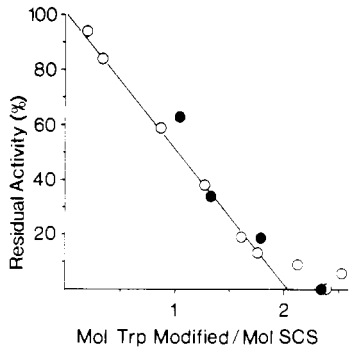

FIGURE 2: Modification of tryptophan residues during reaction of succinyl-CoA synthetase with *N*-bromosuccinimide. Enzyme samples were treated as described in Figure 1. The modified enzyme was analyzed for tryptophan by the methods of Spande and Witkop (1967a) (●) and of Sasaki et al. (1975) (○).

1975). When amino acid analyses of *N*-bromosuccinimide-modified synthetase yielded no methionine sulfone, the possible formation of methionine sulfoxides was considered. The results of this analysis are presented in Table II. It can be seen from these data that, while up to 75% loss of activity was realized, only small quantities of methionine sulfoxide were formed. At a higher *N*-bromosuccinimide level, where 88% of the activity was lost, significant methionine sulfoxide formation did occur. However, the pattern of residue modification would not account for loss of activity as being attributable only to methionine modification. Other data bearing on this point will be presented in the discussion of Figure 4.

A clear-cut relationship between destruction of tryptophan and loss of synthetase activity was seen, as shown in Figure 2. The figure indicates that at 100% loss of activity two tryptophan residues have been modified per $\alpha_2\beta_2$ molecule. This is consistent with the loss of one tryptophan per β-subunit. These data were complemented by those from an experiment in which protein fluorescence was measured before and after treatment with *N*-bromosuccinimide. The results of that experiment are shown in Figure 3. It can be seen that the fluorescence intensity due to tryptophan residues in the chemically modified protein was reduced by 20%. The fact that the fluorescence was not reduced by the anticipated 33% may be due to a lower quantum yield for the *N*-bromosuccinimide-sensitive tryptophans.

Other methods of tryptophan modification have been tried and have given variable results. When *N*-bromosuccinimide was used at pH 4 (Spande & Witkop, 1967b), inactivation was observed but without significant loss of tryptophan residues. *N*-Chlorosuccinimide, which has been used for peptide cleavage of tryptophanyl residues (Schechter et al., 1976), caused inactivation of succinyl-CoA synthetase at pH 7, but insignificant amounts of tryptophan were modified. Even when

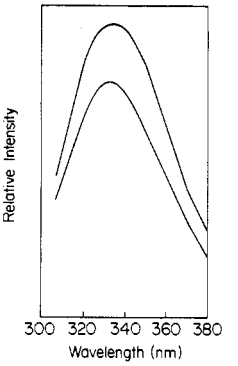

FIGURE 3: Fluorescence emission spectra of unmodified and *N*-bromosuccinimide-modified succinyl-CoA synthetase. Incubation (at a ratio of reagent to enzyme of 20) was carried out, as described in Figure 1, in a final volume of 85 μL. After incubation, 2 μL of 121 mM *N*-acetylmethionine was added to quench the reaction. The solutions were passed through Sephadex G-25 M columns (PD-10; Pharmacia Fine Chemicals) that had been equilibrated with 50 mM Tris-HCl (pH 7.5). The eluates were concentrated in an Amicon pressure filtration concentrator. Protein concentrations were determined by the method of Lowry et al. (1951) and were adjusted to 100 μg/mL with the Tris buffer. The fluorescence spectrum of each sample was taken with excitation at 295 nm. Upper curve, unmodified enzyme; lower curve, modified enzyme.

used at high reagent to enzyme ratios, 2-hydroxy-5-nitrobenzyl bromide (Koshland et al., 1964) had little inhibitory effect on the enzyme. On the other hand, the efficacy of inactivation of the synthetase by 2-nitrophenylsulfenyl chloride at pH 4 (Fontana & Scoffone, 1972) was comparable to that observed with *N*-bromosuccinimide at pH 7. The extent to which tryptophan had been modified in this case has not yet been determined.

ATP-Mg^{2+}, succinate, and desulfo-CoA (analogue of CoA), added at saturating concentrations singly, in pairs, and all together, failed to protect the enzyme against modification by *N*-bromosuccinimide. This was a puzzling result, since quenching of enzyme tryptophan fluorescence by acrylamide has been shown to be affected by the presence of substrates (Prasad et al., 1983a).

Preliminary studies indicated that *N*-bromosuccinimide-modified enzyme (at a ratio of reagent to enzyme of 20) did retain some capacity for phosphorylation, even though residual enzymatic activity was negligible. This suggested that modification of the β-subunit was more critical than that of the α-subunit. In order to determine the effect of tryptophan modification in the β-subunit on the phosphorylation of the α-subunit without the ambiguity that would arise from the simultaneous modification of methionine residues (see Table II), subunits were isolated from enzyme that had been treated at a *N*-bromosuccinimide to enzyme ratio of 10. Under these conditions, modification of methionine residues of the β-subunit had been shown to be negligible (see Table II). In the present case, analysis gave a value of 1.07 tryptophan residue modified/mol of enzyme. If intact tryptophan were required for enzymatic activity, it would be expected that there should be a loss of about 50% of the enzyme activity. Results of refolding experiments with these subunits are described in Figure 4. Whether mixed with unmodified α-subunit or with modified α-subunit, modified β-subunit gave a refolded product that was 50% as active as the unmodified refolded control (Figure 4A). Modified α-subunit and unmodified β-subunit gave a refolded product that was over 80% as active as the control. In the case of phosphorylation (Figure 4B), the mixture of modified β-subunit and unmodified α-subunit gave phosphorylation values close to those obtained with the refolded un-

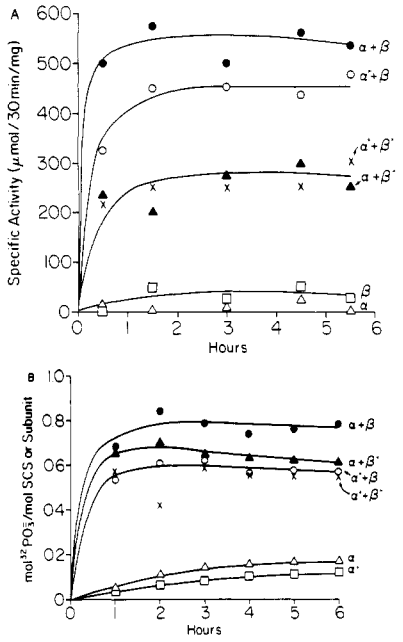

FIGURE 4: Time course of refolding and phosphorylation of subunits obtained from enzyme that had been partially inactivated by *N*-bromosuccinimide. (A) Regain of enzymatic activity. (B) Phosphorylation of refolded enzyme. The protein (44 μg of unmodified or modified α; 56 μg of unmodified or modified β) was contained in 60 μL of 6 M urea–5% acetic acid–0.5 mM dithiothreitol–0.1 mM EDTA. The following additions were made at 0 °C: 21 μL of 3 M Tris-HCl (pH 10.3), 30 μL of 0.413 mM [γ-^{32}P]ATP (specific radioactivity approximately 300 μCi/μmol), and 849 μL of "refolding solution" containing 40 mM Tris-HCl (pH 7.5), 11.3 mM $MgCl_2$, and 11.3 mM dithiothreitol. Incubation was carried out at 23 °C. Samples were removed for enzyme assay and the remainder of the solution was extracted with phenol in order to determine the extent of phosphorylation (Boyer & Bieber, 1967). Unmodified subunits, α and β; modified subunits, α* and β*. Modification was performed at a *N*-bromosuccinimide to enzyme ratio of 10. Enzyme used in this experiment had an original specific activity of 1100 μmol/(min·mg). Other details are described in the text.

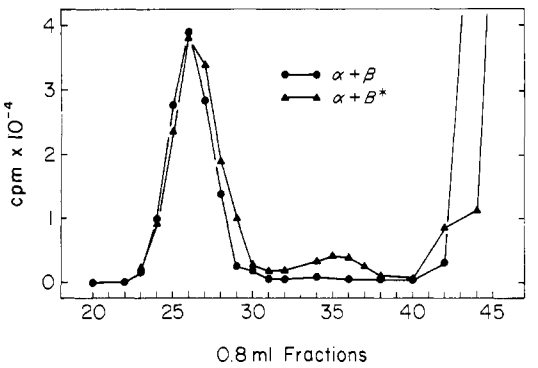

FIGURE 5: Gel filtration of refolded products on Sephadex G-200. Unmodified and modified subunits were incubated as described in Figure 4, but for 4 h. The reaction mixtures (960 μL) were then passed through a Sephadex G-200 column (1 × 80 cm) that had been equilibrated with 50 mM Tris-HCl (pH 7.2)–50 mM KCl–0.1 mM EDTA–0.1 mM dithiothreitol. Fractions were assayed for ^{32}P.

modified control at 1–2 h, although this value dropped slightly over the next 4 h. The rate of phosphorylation of unmodified or modified α-subunit in the presence of modified β-subunit also appeared to be normal. Finally, when modified α-subunit was incubated without β-subunit, phosphorylation proceeded at a near-normal rate, whereas phosphorylation of α-subunit modified at the higher concentration of *N*-bromosuccinimide (ratio of 20) was abolished (data not shown).

It was important to determine if the phosphorylated refolded products were actually $a_2\beta_2$ forms of the enzyme. Pearson and Bridger (1975a) had shown that refolded α- and β-subunits gave a product that was indistinguishable, with respect to molecular size, from the native enzyme. This was confirmed for the hybrid of unmodified α-subunit and modified β-subunit (modified at a ratio of *N*-bromosuccinimide to enzyme of 20) by comparing its elution volume from a Sephadex G-200 column with that of refolded unmodified subunits, as shown in Figure 5. A small peak at fraction 35 indicated the possible presence of free phosphorylated α-subunit. This was verified in an experiment in which only α-subunit was refolded in the presence of [γ-^{32}P]ATP.

Discussion

In order to determine the site(s) of chemical modification of succinyl-CoA synthetase of *E. coli*, the fact that the subunits of the enzyme can be isolated and refolded to yield functional enzyme molecules (Pearson & Bridger, 1975a) can be exploited. Although complete enzymatic activity is not usually regained in refolding, it appears that the regained activity is attributable to molecules that are correctly folded (Wolodko et al., 1981). In the present study, advantage was taken of this knowledge as well as of the fact that the α-subunit of the enzyme is phosphorylated during the catalytic reaction (eq 2a).

N-Bromosuccinimide (at pH 7) appears to be a fairly selective chemical modifying agent of *E. coli* succinyl-CoA synthetase. The data presented here are consistent with the modification of a single tryptophan residue in the β-subunit resulting in inactivation of the enzyme. However, modification of this residue does not appear to interfere significantly with the extent to which the α-subunit in the $\alpha_2\beta_2$ structure is phosphorylated. Early modification experiments (data not presented) were performed at a *N*-bromosuccinimide to synthetase ratio of 20. β-Subunit obtained under these conditions contained one modified tryptophan residue but was able to support the phosphorylation of unmodified α-subunit when refolded with the latter, albeit at a greatly reduced rate. However, the additional modification of 1 mol of methionine in the β-subunit made interpretation of the data difficult. Fortunately, at a ratio of reagent to enzyme of 10, no methionine was oxidized in the β-subunit (Table II). Thus, with β-subunit modified to the extent of only 0.5 tryptophan per molecule, it has been shown that phosphorylation of the refolded enzyme proceeds normally (Figure 4B). Half of the refolded molecules should have been unmodified and exhibited normal phosphorylation rates. The other half of the refolded molecules was modified but also exhibited normal phosphorylation rates; i.e., phosphorylation was essentially completed in the first hour compared with 4 h for the refolded product containing the more extensively modified β-subunit (modified at a *N*-bromosuccinimide to enzyme ratio of 20). Therefore, aside from the loss of catalytic activity that can be ascribed to destruction of a tryptophan residue, it is possible that a methionine residue may be involved sterically in the interaction of the β-subunit with the α-subunit. Modification of such a residue might be expected to alter the affinity of the β-subunit for the α-subunit, resulting in a slower phosphorylation of the latter by ATP. Another possible explanation is that several different methionine residues are fractionally modified, with the net effect being altered interaction between subunits. Clearly, more work will be required to clarify this phenomenon.

It appears, then, that while β-subunit that is modified with respect to one tryptophan residue is catalytically inactive, it is nevertheless able to refold to the extent required for combination with refolded α-subunit to yield a form of the enzyme that is capable of normal interaction with ATP. It is also

conceivable that the β-subunit contributes to the ATP binding domain (Nishimura et al., 1983). By the criterion of gel filtration (Figure 5), refolded enzyme containing modified β-subunit appears to be of normal molecular size.

It had been considered to attempt modification of the isolated β-subunit with *N*-bromosuccinimide. However, unlike the α-subunit, the β-subunit appears to aggregate (unpublished data). Interpretation of chemical modification studies of such heterogeneous molecular species would have been difficult at the outset. Therefore, this approach was not pursued.

Determination of the proximity of the modified tryptophan residue to the active site will require further study. The fact that substrates and the CoA analogue desulfo-CoA did not protect the enzyme against modification by *N*-bromosuccinimide would appear to indicate that the tryptophan is not at the active site. Quite recently, the amino acid sequences of α- and β-subunits of succinyl-CoA synthetase have been deduced by sequencing of the genes of the enzyme in *E. coli* (Buck et al., 1985). The data confirm our finding that there are three tryptophan residues in the β-subunit and none in the α-subunit (Prasad et al., 1983a). Our efforts will now be directed toward determining which of the three tryptophan residues is modified by *N*-bromosuccinimide.

As shown in Tables I and II, α-subunit from enzyme that was modified at a ratio of *N*-bromosuccinimide to enzyme of 10 contained some modified tyrosine and histidine and a small quantity of methionine sulfoxide. Its ability to undergo autophosphorylation or phosphorylation in the presence of the β-subunit was not significantly different from the refolded control. However, at a reagent to enzyme ratio of 20, a significant increase of methionine sulfoxide in the α-subunit was observed (Table II). In addition, the subunit was inactive with respect to autophosphorylation (data not shown). In this case, it may be of interest to attempt modification of isolated α-subunit. This may provide a more clear-cut approach to study structure–function relationships in this subunit.

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Registry No. L-Trp, 73-22-3; succinyl-CoA synthetase (ADP forming), 9080-33-5.

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