Intrinsic Fluorescence of Succinyl-CoA Synthetase and Four Tryptophan Mutants. Tryptophan 76 and Tryptophan 248 of the β-Subunit Are Responsive to CoA Binding†

Jonathan S. Nishimura,* Craig J. Mann, Jesse Ybarra, Theresa Mitchell, and Paul M. Horowitz
Department of Biochemistry, The University of Texas Health Science Center at San Antonio, San Antonio, Texas 78284-7760
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ABSTRACT: Previous studies showed that modification of an average of one of the three tryptophan residues of succinyl-CoA synthetase of *Escherichia coli* abolished enzyme activity, but did not prevent phosphorylation of the enzyme by ATP [Ybarra, J., Prasad, A. R. S., & Nishimura, J. S. (1986) *Biochemistry 25*, 7174–7178]. In the present study, single mutations in which each of the three tryptophans (β-Trp^{43}, β-Trp^{76}, and β-Trp^{248}) has been changed to phenylalanine (designated W43F, W76F, and W248F) have been accomplished by the technique of site-directed mutagenesis and the mutant proteins isolated. In addition, a double mutant in which β-Trp^{43} and β-Trp^{248} were changed to phenylalanines (W43,248F) has also been isolated. Each of the mutant enzymes was practically as active as wild type. Since the emission spectrum of β-Trp^{76} reflected a low fluorescence intensity for this residue, it was possible to obtain the emission spectrum of each tryptophan residue by using W43F, W248F, and W43,248F. From the positions of the emission maxima and the results of iodide quenching of fluorescence, it was deduced that β-Trp^{248} is a surface residue, β-Trp^{43} is buried, and β-Trp^{76} is intermediate in location. Coenzyme A, but no other substrate, protected the fluorescence of β-Trp^{76} and β-Trp^{248}, but not of β-Trp^{43}, against quenching by acrylamide. These results are consistent with an interaction between β-Trp^{76} and β-Trp^{248} and the binding site for CoA.

Succinyl-CoA synthetase (SCS)[1] of *Escherichia coli* catalyzes the reaction (Nishimura & Grinnell, 1972; Bridger, 1974; Nishimura, 1986)

$$\text{succinate} + \text{CoA} + \text{ATP} \leftrightarrow \text{succinyl-CoA} + \text{ADP} + \text{P}_i \quad (1)$$

The enzyme has an $\alpha_2\beta_2$ subunit structure (Bridger, 1971) with two active sites that are located at points of contact between the α- and β-subunits (Bridger, 1974; Vogel & Bridger, 1982; Collier & Nishimura, 1978). The sequence of both subunits has been deduced from the DNA sequence of the genes (Buck et al., 1985). Available evidence indicates that α-His^{246} is the site of catalytic phosphorylation (Bridger, 1974; Buck et al., 1985) and β-Cys^{325} is located close to the CoA binding site (Mann et al., 1989).

Each of the β-subunits contains three tryptophan residues. There are no tryptophans in the α-subunit (Prasad et al., 1983). Other experiments conducted in this laboratory with SCS indicated that the fluorescence quenching properties of at least one tryptophan residue are affected by substrate binding (Prasad et al., 1983). Chemical modification of the enzyme with *N*-bromosuccinimide showed that the modification of, on the average, one tryptophan residue per β-subunit resulted in complete loss of enzymatic activity (Ybarra et al., 1986). However, phosphorylation of the modified enzyme by ATP still occurred, indicating a possible lesion of the active site in the region of CoA or succinate binding.

In the present investigation, each of the three tryptophan residues of the enzyme, β-Trp^{43}, β-Trp^{76}, and β-Trp^{248}, has been subjected to oligonucleotide-directed replacement by phenylalanine. In addition, a double mutant, in which both β-Trp^{43} and β-Trp^{248} were changed to phenylalanines, has been prepared. Enzymatic activity is retained in each of the purified mutant enzymes. Intrinsic fluorescence and fluorescence quenching experiments reveal that β-Trp^{76} and β-Trp^{248} are both responsive to the binding of CoA to SCS, but β-Trp^{43} is not.

Experimental Procedures

Materials

Bacterial Strains and Plasmids. *E. coli* strain DH5αF′ (φ80d*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17(r_{k^-},m_{k^+}) *sup*E44 λ-*thi*-1 *gyr*A *rel*A1) was obtained from Bethesda Research Laboratories. Strain CJ236 *dut*1 *ung*1 *thi*1 *rel*A1 pCJ105[Cm^r]) was obtained from International Biotechnologies, Inc. *E. coli* strain TK3D18 (Δ[*kdp*-suc]D18 Δ[*gal-bio*]), which does not express the *suc*C and *suc*D genes (J. R. Guest, personal communication), was a gift from Dr. Wolfgang Epstein, University of Chicago. Plasmid pGS131 was obtained from Dr. John R. Guest, University of Sheffield. This plasmid contains the *suc*C and *suc*D genes that code for the α- and β-subunits of succinyl-CoA synthetase on a 4.5 kilobase pair *Bgl*II fragment inserted into the *Bam*HI site of pBR322 (Buck et al., 1985). The Bluescript plasmid pSKM was obtained from Stratagene Cloning Systems. Bacteriophage M13mp18 and mp19 were obtained from Bethesda Research Laboratories.

Enzymes and Nucleotides. Restriction enzymes were obtained from Bethesda Research Laboratories and New England Biolabs. T4 DNA ligase and T4 polynucleotide kinase were obtained from Bethesda Research Laboratories. Se-

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* To whom correspondence should be addressed.

[1] Abbreviations: SCS, succinyl-CoA synthetase; W43F, tryptophan residue at position 43 changed to phenylalanine, one of several similar notations.

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Table I: Properties of Wild-Type SCS and Mutants

enzyme	specific activity: μmol 30 min^{-1} mg^{-1} [a]	specific activity: μmol min^{-1} mg^{1} [b]	$A_{280nm}^{1.0mg/mL}$: Lowry method	$A_{280nm}^{1.0mg/mL}$: biuret method
wild type	850	37	0.51	0.51
W43F	780	34	0.42	0.44
W76F	810	35	0.38	0.38
W248F	900	39	0.42	0.47
W43,248F	750	32	0.42	0.40

[a] Units of Kaufman (1955). [b] Units of Bridger et al. (1969).

quenase DNA polymerase was purchased from United States Biochemical Corp. Deoxynucleotides and dideoxynucleotides were purchased from Pharmacia P-L Biochemicals. [α-^{35}S]Thio-dATP was purchased from New England Nuclear.

Oligonucleotides. Oligonucleotides were synthesized by Dr. Robert McGregor, Texas A&M University, and were then purified by gel electrophoresis (Atkinson & Smith, 1984). The following oligonucleotides (altered bases underlined) were prepared: (a) W43F mutagenic oligonucleotide, dTGCCGGTCCGTTCGTAGTGAAATG; (b) W76F mutagenic oligonucleotide, dTGCAGAAAACTTCCTGGGCAAGCG; (c) W248F mutagenic oligonucleotide, dGGCTGCACAGTTCGAACTGAACTA; (d) sequencing primer for W43F (40 base pairs upstream) and W76F (140 base pairs upstream), dTGTACTACTCCGCGCGAA; (e) sequencing primer for W248F (40 base pairs upstream), dCGCGAAATGCGTGACCAG.

Methods

Electrophoresis. Restriction enzyme digests of plasmid or phage DNA were analyzed by electrophoresis on agarose submarine gels in Tris–borate–EDTA buffer and visualized by ethidium bromide staining (Maniatis et al., 1982).

Oligonucleotide-Directed Mutagenesis and Expression of SCS Mutants. The procedures used have been described recently (Mann et al., 1989). The double-tryptophan mutant was made by first introducing a single mutation into the wild-type DNA. The resulting mutant DNA was then purified and used to introduce the second mutation.

Purification of Succinyl-CoA Synthetases. The wild-type and mutant enzymes were purified by methods that have been described previously (Grinnell & Nishimura, 1969; Bowman & Nishimura, 1975).

Protein Determination. Protein concentrations were measured by the method of Lowry et al. (1951) and by the biuret method (Gornall et al., 1949), using purified succinyl-CoA synthetase as standard.

Enzyme Assays. Succinyl-CoA synthetase activity was assayed by a modification (Grinnell & Nishimura, 1969) of a previously described method (Kaufman, 1955).

Fluorescence Measurements of Native and Denatured Enzymes. The intrinsic fluorescence spectra of wild-type and mutant enzymes were measured after the enzymes were first diluted with 0.1 M sodium phosphate, pH 7.4, to a final protein concentration of 0.1 mg/mL (Lowry et al., 1951). Spectra were recorded on a SLM Aminco SPF-500 spectrofluorometer and corrected by using a buffer blank. Fluorescence spectra of the denatured protein were measured after the enzymes were diluted with 6 M guanidine hydrochloride in 0.1 M sodium phosphate, pH 7.4, to a final protein concentration of 0.1 mg/mL. Samples were incubated overnight at 4 °C. Spectra were recorded as before after correction with respect to a buffer blank containing 6 M guanidine hydrochloride.

Quenching of Fluorescence by Acrylamide and Iodide. Fluorescence quenching was determined and analyzed by the method previously described (Prasad et al., 1983).

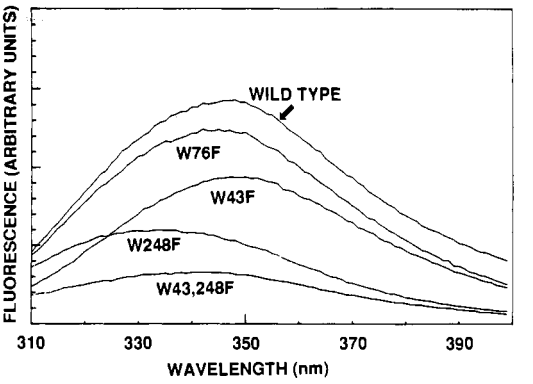

FIGURE 1: Fluorescence emission spectra of SCS and its tryptophan mutants. The protein was contained in 0.1 M sodium phosphate, pH 7.5. The excitation wavelength was 295 nm.

Table II: Fluorescence Emission Maxima of Enzyme Forms

protein	native (nm)	denatured (nm)
wild type	346–348	355
W43F	347–350	355
W76F	345	355
W248F	330–335	355
W43,248F	340–345	355

Results

The specific activities of the tryptophan mutants of SCS were not greatly different from that of the wild-type enzyme, as shown in Table I. Nevertheless, the mutants in which β-Trp^{43} was replaced, viz., W43F and W43,248F, were somewhat less active than wild-type enzyme. The A_{280nm} values for solutions of 1.0 mg/mL were expectedly lower than those of wild type for the single mutants. It is interesting that, according to the protein method of Lowry et al. (1951), the double mutant had an A_{280nm} value that was equal to those of two single mutants (W43F and W248F) and higher than that of the third (W76F). With the biuret method the A_{280nm} value of the double mutant was still slightly higher than that of W76F, but somewhat lower than that of either W43F or W248F.

The tryptophan fluorescence emission spectra of the mutant proteins were compared with the emission spectrum of wild-type SCS (Figure 1) (see also Table II). Surprisingly, the emission spectrum of W76F (Phe in place of Trp at residue β-76) was very similar to that of wild-type enzyme, with only a slight difference in fluorescence intensity. The fluorescence intensity of W43F was approximately two-thirds that of wild-type SCS, as expected, with a slight red shift of the peak. However, the fluorescence intensity of W248F was reduced by 60% compared with wild type, with a significant blue shift of the peak. The fluorescence intensity of W43,248F, the double mutant, attributable to β-Trp^{76}, was also lower than expected, but involved only a slight blue shift in the emission peak. These apparent inconsistencies were clarified when the tryptophan emission spectra were measured by using protein samples that had been denatured in 6 M guanidine hydrochloride (Figure 2). Thus, the fluorescence intensity for each of the three single mutants was approximately two-thirds, and that of the double mutant approximately one-third, of wild type. It is apparent that β-Trp^{76} fluorescence is significantly quenched in the wild-type enzyme and that W248F and W43F contain β-Trp^{43} and β-Trp^{248}, respectively, as dominant tryptophan fluorophores. Thus, the potential exists to study the contribution of the individual tryptophan residues by using the

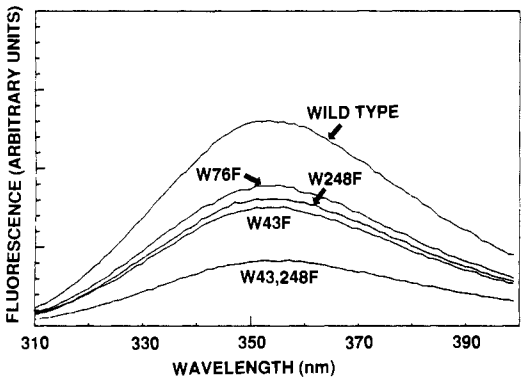

FIGURE 2: Fluorescence emission spectra of SCS and its tryptophan mutants in 6 M guanidine hydrochloride. The procedure was as described in Figure 1, except that 6 M guanidine hydrochloride was added to all samples.

Table III: Effects of Substrates on SCS Tryptophan Quenching by Acrylamide[a]

		K_{SV} (M^{-1}) in presence of substrates				
enzyme	Trp present	no substr	CoA (1 mM)	ATP (5 mM)	Succ (5 mM)	ADP (5 mM)
wild type	43, 76, 248	5.9	4.5	6.3	6.0	6.4
W 43F	76, 248	7.1	6.1	7.7	7.5	6.9
W76F	43, 248	6.8	5.1	7.2	6.3	6.9
W248F	43, 76	4.9	4.8	5.0	5.1	5.2
W43,248F	76	6.2	5.0	6.2	6.2	6.2

[a] The samples contained SCS (0.1 mg/mL) in 0.1 M sodium phosphate (pH 7.5) and acrylamide in increasing concentrations from 0 to 0.5 M. Concentrations of substrates are given. F_0/F (where F and F_0 are the fluorescence intensities in the presence and absence of quencher, respectively) were plotted vs acrylamide concentration. The slope of the plot, K_{SV} (Stern–Volmer constant), was then determined.

mutated proteins while retaining full function.

Next, the effect of substrates on quenching of tryptophan fluorescence was examined (Table III). Stern–Volmer constants were obtained for acrylamide quenching in the absence and presence of the substrates CoA, ATP, succinate, and ADP. CoA protected wild-type enzyme against quenching, while the other substrates actually enhanced quenching. However, quenching of W248F, which lacks β-Trp^{248}, was not affected by CoA. On the other hand, quenching of W43F and W76F, both of which contain tryptophan at position 248 of the β-subunit, was protected against by CoA, but no other substrate. These data are consistent with the hypothesis that β-Trp^{248} is either at or near the active site or its environment is affected by binding of CoA to the active site. The data in Table III also indicate that the fluorescence of β-Trp^{248} masks the less apparent fluorescence of β-Trp^{76}. Thus, quenching of the fluorescence of the double mutant, which contains only β-Trp^{76}, was also protected against by CoA, but not by any other substrate.

Addition of iodide affected fluorescence of W248F to a much lesser degree than that of wild-type enzyme, W43F and W76F (Table IV). These results indicate that the fluorescence of β-Trp^{248}, but not that of β-Trp^{43}, is quenched by iodide and that β-Trp^{248} is probably located at or near the surface of the molecule. This is in keeping with the longer λ_{max} of W43F in which β-Trp^{248} makes the major contribution to fluorescence (Burstein et al., 1973). Quenching of tryptophan fluorescence by cesium ion gave similar results (results not shown), indicating that a cation–anion interaction was not involved in the quenching by iodide ion. In the case of the double mutant, W43,248F, fluorescence was only slightly quenched by either iodide or cesium ion (data not shown), indicating that β-Trp^{76} is not in contact with solvent. This is consistent with the shorter λ_{max} in the spectrum of W43,248F (Burstein et al., 1973).

Table IV: Iodide Quenching of Tryptophan Fluorescence of SCS and Mutant Enzymes[a]

enzyme	Trp present	K_{SV} (M^{-1})
wild type	43, 76, 248	1.10
W43F	76, 248	0.92
W76F	43, 248	1.30
W248F	43, 76	0.55

[a] K_{SV} values were obtained in the presence of iodide, added at 0–0.2 M. Other conditions were as described in Table III.

Discussion

Previously, we demonstrated that chemical modification of one tryptophan residue of SCS led to complete loss of enzymatic activity (Ybarra et al., 1986). However, the modified enzyme was still able to undergo phosphorylation, albeit at a slower rate. Thus, it was anticipated that mutation of each of the tryptophan residues in SCS would yield at least one enzyme form that would differ significantly in activity from wild type. Contrary to this expectation, the activity of each of the three mutants was not significantly different from that of wild type. Apparently, phenylalanine is sufficiently like tryptophan so that this replacement does not destabilize the protein at the site(s) formerly occupied by tryptophan. However, the chemical modification of tryptophan in SCS by *N*-bromosuccinimide (Ybarra et al., 1986) appears to interfere with substrate binding (perhaps that of CoA). Oxidation of a tryptophan residue may cause an increase in the size of the side chain of this amino acid and thus induce an unfavorable conformational change in the enzyme. In this connection, we have shown that whereas chemical modification of a sensitive sulfhydryl group led to complete inactivation of SCS, site-directed mutagenesis of β-Cys^{325} to a glycine residue had little effect on activity or stability properties of the enzyme (Mann et al., 1989). On the other hand, substitution of β-Cys^{325} with asparagine or threonine, amino acids with bulkier side chains, gave rise to enzyme forms that were less active and less stable thermally (Mann et al., unpublished results).

The three tryptophan residues of SCS have distinctly different fluorescence properties. β-Trp^{76} fluorescence is quenched significantly. There are several possible reasons for this, including proximity of the indole side chain to a charged amino acid residue (Beechem & Brand, 1985). Since the fluorescence of β-Trp^{76} was suppressed, examination of W248F and W43F permitted determination of the fluorescence emission spectra of β-Trp^{43} and β-Trp^{248}, respectively. This analysis revealed that, in all likelihood, the microscopic environments of these amino acid residues are markedly different. In fact, it would appear from the position of its emission peak that β-Trp^{248} is located at or near the surface of the enzyme molecule, whereas β-Trp^{43} is "buried". Comparison of the fluorescence spectra of W43F, W248F, and W43,248F suggests that the microscopic environment of β-Trp^{76} is intermediate between that of β-Trp^{43} and β-Trp^{248}. A more detailed comparison was difficult because of the low fluorescence intensity of β-Trp^{76}.

Previous studies conducted in this laboratory led to the conclusion that the presence of the substrate CoA protects at least one tryptophan residue in SCS against quenching by acrylamide and iodide (Prasad et al., 1983). The data described herein would appear to indicate that both β-Trp^{76} and β-Trp^{248} are involved in this interaction. The relatively low fluorescence of β-Trp^{76} and the availability of W43F and W248F have made it possible to study each of these tryptophan residues independently of each other. In the present study,

it has been shown that CoA protects β-Trp^{248} fluorescence against quenching by acrylamide but not that of β-Trp^{43}. This result suggests an association of β-Trp^{248} with the binding site of CoA. A similar association of the latter with β-Trp^{76} is also indicated. The results of iodide and cesium ion quenching indicate that β-Trp^{248} is probably a surface residue. The location of the tryptophan fluorescence emission peak at longer wavelengths is also consistent with this notion. The fact that the fluorescence of β-Trp^{76} was not quenched by either iodide or cesium ion does not diminish the argument alluded to above that this residue may be intermediate with respect to being buried or located toward the surface of the enzyme. Since its λ_{max} is less than 345 nm, β-Trp^{76} may be expected to have restricted access to mobile solvent.

Recent work in this laboratory has shown that β-Cys^{325} is located at the CoA binding site (Mann et al., 1989). Previously, it had been shown that α-His^{246} is the site of phosphorylation of SCS (Bridger, 1974; Buck et al., 1985). Thus, if β-Trp^{248} is positioned close to β-Cys^{325} and α-His^{246}, it would suggest the involvement of the C-terminal domains of the α- and β-chains in the active site of SCS. The identity of a second cysteine residue of the α-subunit bearing a sulfhydryl group that can be chemically cross-linked to β-Cys^{325} is now under investigation. Clearly, knowledge of the three-dimensional structure of SCS would be highly desirable. The enzyme has been crystallized and some X-ray diffraction data have been published (Wolodko et al., 1984).

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