Sensitivity of *Escherichia coli* Succinyl-CoA Mutants at Trp<sup>676</sup> to Clostripain and to Trypsin

ADP and ATP PROTECT AGAINST CLEAVAGE BY CLOSTRIPAIN AT ARG<sup>390</sup>*

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Mutant forms of *Escherichia coli* succinyl-CoA synthetase, W76F (Trp<sup>676</sup> replaced by Phe) (Nishimura, J. S., Mann, C. J., Ybarra, J., Mitchell, T., and Horowitz, P. M. (1990) *Biochemistry* 29, 862–865), and W43,76,248F (all three Trp replaced by Phe) were found to be more sensitive to proteolysis by clostripain than the wild-type enzyme or other Trp mutant proteins. Like wild-type enzyme, sensitivity to trypsin was apparent when the enzyme forms were in the dephosphorylated state. Sensitivity to clostripain was the same, whether mutant or wild-type forms were in the phosphorylated or dephosphorylated state. The substrates ADP and ATP both protected the enzymes against inactivation by clostripain, with dissociation constants for protection of W76F of 33 and 125 μM, respectively. Polycrylamide gel electrophoresis of clostripain digests revealed preferential digestion of the β-subunit and the appearance of 40- and 31-kDa species, with amino termini corresponding to residues 15 and 81, respectively, of the β-subunit. Mutagenic replacement of Arg<sup>390</sup>, but not Arg<sup>141</sup>, with Lys resulted in an enzyme that was as resistant to clostripain as wild-type enzyme. These results suggest that Arg<sup>390</sup> is the principal site of inactivation by clostripain and may be involved in the binding of ADP and ATP to succinyl-CoA synthetase.

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Succinyl-CoA synthetase of *Escherichia coli* catalyzes the reaction shown in Equation 1.

\[
\text{ATP(GTP) + succinate } \leftrightarrow \text{ ADP(GDP) + Pi + succinyl-CoA} \quad (\text{Eq. 1})
\]

The reaction proceeds via a phosphorylated enzyme intermediate (1, 2). The subunit structure of the enzyme is (αβ)<sub>2</sub>, with phosphorylation (see Equation 2) occurring on the α-subunit at points of contact with the β-subunit.

\[
\text{ATP + enzyme } \leftrightarrow \text{ ADP + P-enzyme} \quad (\text{Eq. 2})
\]

Subsequent covalent steps in the mechanism of the enzymatic reaction are believed to proceed as described in Equations 3 and 4.

\[
\text{P-Enzyme + succinate } \leftrightarrow \text{ [succinyl-P]enzyme} \quad (\text{Eq. 3})
\]

\[
\text{[Succinyl-P]enzyme + CoA } \leftrightarrow \text{ succinyl-CoA + P + enzyme} \quad (\text{Eq. 4})
\]

The β-subunit provides at least part of the CoA-binding site (3) and probably the succinate-binding site (4). The cloning and sequencing of the subunits of succinyl-CoA synthetase (5) have provided a means by which the roles of specific amino acid residues identified by chemical modification and other methods can be studied.

Chemical modification studies have revealed that oxidation of the average 1 tryptophan residue/αβ monomer is sufficient to destroy enzymatic activity but not phosphorylation capacity (6). More recent studies conducted in this laboratory have shown that each of the 3 tryptophan residues in succinyl-CoA synthetase can be replaced by phenylalanine without significant change in enzyme activity (7). These studies also indicated that Trp<sup>676</sup> and Trp<sup>248</sup> are probably located at or near the enzyme surface, whereas Trp<sup>43</sup> is likely a buried residue. In these studies, Trp residues were changed to phenylalanines by the technique of site-directed mutagenesis. Fluorescence quenching experiments with these mutants and with a double mutant, W43,248F<sup>†</sup>, furnished evidence that, in all likelihood, Trp<sup>676</sup> and Trp<sup>248</sup> interact with the CoA-binding site. None of the mutants studied displayed significant differences in activity or stability from wild-type enzyme. Quite recently, we have prepared and isolated the triple mutant, W43,76,248F which is also an active enzyme.

In the present work, we show that W76F and W43,76,248F were considerably more susceptible than wild-type or other tryptophan mutants to proteolysis by clostripain, a protease that splits peptide bonds preferentially at arginine residues. In the dephosphorylated state, like wild-type enzyme, the Trp<sup>676</sup> mutant enzymes were more susceptible to proteolysis by trypsin. ADP and ATP protected W76F, W43,76,248F, and wild-type synthetase, either in the phosphorylated or dephosphorylated state, against clostripain inactivation. During the early phases of clostripain inactivation, the β-subunit was preferentially digested by clostripain, with the initial critical cleavage reaction appearing to occur at either Arg<sup>141</sup> or Arg<sup>390</sup>. Mutagenesis of Arg<sup>390</sup>, but not of Arg<sup>141</sup>, to Lys protected W76F against clostripain. These data suggest that Arg<sup>390</sup> is the principal site of cleavage and inactivation by clostripain and may be involved in the binding of ADP and ATP to SCS.

**EXPERIMENTAL PROCEDURES**

**Materials**

**Wild-type and Mutant Enzymes**—These were prepared as described previously (7). The specific enzyme activities of the species

<sup>†</sup>The single-letter code for amino acids is used to designate the mutant. The first letter denotes the amino acid present in the wild-type enzyme at the number position(s) indicated. The final letter denotes the amino acid present in the mutant enzyme at this(these) position(s). For multiple mutants, more than one number is indicated.
studied, as determined in micromoles (30 min-mg)-1 under "Methods," were: wild-type enzyme, 1090; W43F, 780; W76F, 650; W248F, 900; W43,248F, 750; W43,76,248F, 808; W76F, R14K, 960; and W76F, R80K, 640. Site-directed mutagenesis is discussed under "Methods." Other Reagents—ADP, ATP, clostripain (EC 3.4.22.8), and N-p-tosylphenylalanilinyl chloromethyl ketone (TPCK)2-trypsin was a product of Worthington. All other chemicals were of reagent grade and were obtained from commercial sources.

Methods

Oligonucleotide-directed Mutagenesis and Expression of Trypsin Mutants—The procedures used have been described previously (7, 8) and involved a modification of the method of Kunkel et al. (9) in which uracil-containing single-stranded mp19-SCS template was primed with phosphorylated mutagenic oligonucleotide. Following primer extension and ligation, the closed circular DNA was used to transfected E. coli JM 107 competent cells, according to the procedure of Hanahan (10). Plaques picked at random were screened by the dioxyde sequencing procedure (11), using as primers the sequencing oligonucleotides described below. One mutant was selected and plaque-purified to ensure clonal purity. Double-stranded and single-stranded DNA were prepared from this mutant. The single-stranded DNA was sequenced as before to confirm the sequence at the mutagenic sites. The double-stranded DNA was restricted with either HincII and BamHI. The resulting 2.4-kilobase pair fragment was recloned into the HindIII/BamHI fragment of pSKM to produce the desired DNA primers. Oligonucleotide-directed mutagenesis (16) of the triple mutant W43,76,248F was prepared by mutation of the DNA from W43,248F. Arginine mutants of W76F were prepared by the use of the following oligonucleotides (altered bases underlined): for W76F, R14K, dACTTTTTGCCeTATGACGTATCA. Mutations were confirmed by sequencing of double-stranded DNA isolated from transformed E. coli TK3D18 cells, using the following sequencing primers: for W76F, R14K, dTTCGGTCTACGGTTTAAA which primes 71 nucleotides upstream from the first nucleotide in the mutagenic region; for W76F, R80K, dTGTACATTCCGGCGCGAA which primes 147 nucleotides upstream from the first nucleotide in the mutagenic region.

Purification of Succinyl-CoA Synthetases—Transformed E. coli TKD318 cells were grown as described (8). The wild-type and mutant enzymes were purified by methods that have been described previously (12, 13).

Assay of Succinyl-CoA Synthetase Activity—This was performed by a modification (12) of a previously described method (14). Succinyl-CoA formation is followed by its conversion to succinic hydrazide.

Protein Determination—Protein concentrations were measured by the method of Gornall et al. (15) using purified succinyl-CoA synthetase as standard.

Inactivation of Succinyl-CoA Synthetase and Mutants with Trypsin—The incubation solutions contained 50 mM Tris-HCl, pH 7.2, 50 mM KCl, 5 mM MgCl2, and 1.53 μM succinyl-CoA synthetase. Incubation was carried out for 15 min at 37 °C in the absence ("Native") and presence ("Dephosphorylated") of 200 μM ADP, in a final volume of 550 μL. Then, 20 μL of 0.01% N HCl containing TPCK-treated trypsin (to give a final concentration of 0.42 μM) were added, followed by incubation at 24 °C. At various times over a period of 120 min, 8 μL samples were removed for assay of synthetase activity. Values of the pseudo-first-order rate constant for inactivation were obtained as described by Moffet et al. (16).

Dephosphorylation and Phosphorylation of Succinyl-CoA Synthetase and Mutants for Clostripain Digestion—This was performed according to Bowman and Nishimura (13). In this method, native enzymes were incubated with succinyl-CoA synthetase and the dephosphorylated enzyme is separated from ligands by gel filtration on Sephadex G-50 in 0.1 M Tris-HCl, pH 7.5. Phosphorylated succinyl-CoA synthetase was prepared by incubating native enzyme with 2 mM ATP, 5 mM MgCl2 for 20 min at 37 °C. Ligands were removed from enzyme by gel filtration. Isoelectric focusing was carried out as described by Mann et al. (17) to determine how much dephosphorylated, monophosphorylated, and unphosphorylated protein was present.

Assay of Clostripain—The method involves the hydrolysis of the synthetic substrate N-benzyloxycarbonyl arginine ethyl ester which is monitored at 253 nm, using a molar absorptivity difference of 3000 M-1 cm-1 at 18°C (18). For activation, clostripain (0.8 mg/ml) was incubated in a solution containing 20 mM sodium phosphate, pH 7.5, 2.5 mM dithiothreitol, and 1 mM CaCl2, for 17 h at 4 °C.

Polyacrylamide Gel Electrophoresis by the Method of Laemmli (19)—Samples drawn from clostripain digest of mutant and wild-type succinyl-CoA synthetase were subjected to polyacrylamide gel electrophoresis, as described by Laemmli (19), on a 16-cm wide × 14-cm long × 1.5-mm thick slab, using 16% acrylamide, 3% bisacrylamide. The gel was stained overnight in 0.025% Coomassie Brilliant Blue R (dissolved in 40% methanol, 10% acetic acid) and destained by diffusion of excess dye in 5% methanol, 7.5% acetic acid.

Densitometric Scanning of Stained Polyacrylamide Gel—This was performed with a BioImage Visage model 110 digital image processing instrument, using 1-D Scan software.

Polyacrylamide Gel Electrophoresis by the Method of Schagger and Von Jagow (20)—The native and the three tryptophan mutants of E. coli TK3D18 cells which are null with respect to succinyl-CoA synthetase (5). The DNA of the triple mutant W43,76,248F was prepared from Sigma. DNA was digested with HindIII and BamHI. The resulting 2.4-kilobase pair fragment was recloned into the HindIII/BamHI fragment of pSKM to produce the desired DNA primers. Oligonucleotide-directed mutagenesis (16) of the triple mutant was performed by mutation of the DNA from W43,248F. Arginine mutants of W76F were prepared by the use of the following oligonucleotides (altered bases underlined): for W76F, R14K, dACTTTTTGCCeTATGACGTATCA. Mutations were confirmed by sequencing of double-stranded DNA isolated from transformed E. coli TK3D18 cells, using the following sequencing primers: for W76F, R14K, dTTCGGTCTACGGTTTAAA which primes 71 nucleotides upstream from the first nucleotide in the mutagenic region; for W76F, R80K, dTGTACATTCCGGCGCGAA which primes 147 nucleotides upstream from the first nucleotide in the mutagenic region.

Digestion of Wild-type Succinyl-CoA Synthetase and Mutant Proteins with Trypsin—It had been demonstrated previously that the presence of ATP-Mg2+ had a marked protective effect against inactivation of succinyl-CoA synthetase by trypsin (18). It was also shown that this protective effect was due to phosphorylation of unphosphorylated catalytic histidine residues of the enzyme. On the other hand, removal of phosphoryl groups by the addition of ADP-Mg2+ caused a liability of the enzyme that was related to the degree of dephosphorylation. One interpretation of this observation is that dephosphorylation brings about a conformational change which results in exposure of a peptide bond involving an arginine or lysine residue of the enzyme to tryptic cleavage. On the premise that small conformational changes elicited by mutation of tryptophan residues might also bring about altered exposure of sites to solvent and, thus, facilitate proteolysis, each of five tryptophan mutants, the single mutants W43F, W76F, and W248F and the double mutant W43F, W248F (7), as well as the triple mutant (W43,76,248F), was incubated with trypsin (TPCK-treated trypsin was actually employed) in the native (phosphorylated) and dephosphorylated state. The results, shown in Table I, indicate that each of the enzymes, in the native state (with the exception of the triple mutant), was fairly resistant to inactivation by trypsin. However, in the dephosphorylated state, both Trp76 mutants were more sensitive to trypsin than wild-type enzyme or any of the other tryptophan mutants tested. Isoelectric focusing revealed that virtually all of the native enzyme was in the dephosphorylated and monophosphorylated state and that phosphoryl groups were virtually absent in the dephosphorylated enzyme. This result is consistent with the observations made by Mann et al. (17) with wild-type enzyme. As men-
TABLE I

Susceptibility of succinyl-CoA synthetases to inactivation by trypsin

The incubation conditions and method for determination of values for k, the pseudo-first-order rate constant of inactivation, are described under Methods. Dephosphorylation was achieved by incubation of enzyme with ADP.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Status</th>
<th>k (min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>Native</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td>Dephosphorylated</td>
<td>0.025</td>
</tr>
<tr>
<td>W43F</td>
<td>Native</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td>Dephosphorylated</td>
<td>0.027</td>
</tr>
<tr>
<td>W76F</td>
<td>Native</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td>Dephosphorylated</td>
<td>0.048</td>
</tr>
<tr>
<td>W248F</td>
<td>Native</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Dephosphorylated</td>
<td>0.017</td>
</tr>
<tr>
<td>W43,248F</td>
<td>Native</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td>Dephosphorylated</td>
<td>0.026</td>
</tr>
<tr>
<td>W43,76,248F</td>
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</tr>
<tr>
<td></td>
<td>Dephosphorylated</td>
<td>0.077</td>
</tr>
</tbody>
</table>

Effects of ADP on the Activity of Clostripain—To more clearly define the apparent protective effects of ATP and ADP against clostripain inactivation of either W76F or W43,76,248F, the samples of mutant and wild-type enzyme were subjected to phosphorylation or dephosphorylation conditions followed by removal of the responsible ligands, as described under “Methods,” prior to treatment with the protease. Because of the greater sensitivity of the triple mutant to clostripain, half the amount of clostripain was employed in the experiment. The results, described in Fig. 3, show clearly that each of the enzymes was cleaved at the same rate, in either its phosphorylated or dephosphorylated state. The latter result indicates that the Trp76 mutants are conformationally different from wild-type enzyme, regardless of their phosphorylation state. Neither ADP nor ATP had any effect on the activity of clostripain, as demonstrated in control experiments in which clostripain activity on the synthetic substrate N-benzoylarginine ethyl ester (see “Methods”) was measured.

SDS-Polyacrylamide Gel Electrophoresis, According to the Method of Laemmli, of Clostripain Digests of W76F—Progress of protection of both enzymes was also observed with 1 mM ADP.

Determination of Apparent Dissociation Constants of ADP and ATP for Protection of W76F against Inactivation by Clostripain—It was also of interest to determine that protection was a reflection of the affinity of ADP or ATP for the enzyme. The data in Fig. 2 show that low concentrations of ADP protected W76F against clostripain, with an apparent KD for ADP of 33 μM. This compares favorably with a KD for ADP of 68.3 μM obtained by rate of dialysis measurements (16). Thus, it would appear that ADP protection against clostripain action is associated with relatively high affinity binding of the nucleotide to the enzyme. The apparent KD for ATP was 125 μM, reflecting a lower affinity of ATP than ADP for the enzyme. This is not surprising, since ATP is known to bind poorly to the phosphorylated form of the enzyme (13).
The digestion of W76F by clostripain was monitored by electrophoresis, as shown in Fig. 4. Clearly, the β-subunit was digested more rapidly than the α-subunit. In fact, the loss of β appeared to mirror the loss of succinyl-CoA synthetase enzymatic activity, while α appeared to remain intact until approximately 80% of the enzymatic activity had disappeared. Faint bands that migrated between the subunits, indicating possible intermediate proteolytic products of the β-subunit, were not well resolved by this gel system. Another electrophoresis and staining system (see below) was used for detection of intermediate products. In parallel studies with wild-type synthetase, it appeared that β-subunit was also preferentially digested, albeit at a slower rate.

**FIG. 3.** Effect of phosphorylation state on susceptibilities of Trp76 mutants and wild-type enzyme to clostripain. Incubations were performed as described in Fig. 1, except that the clostripain concentration was reduced by half. Conditions for phosphorylation by ATP-Mg²⁺ and dephosphorylation by succinate plus CoA in the presence of hydroxylamine are described under "Methods." Phosphorylated W43,76,248F Trp mutants and wild-type enzyme to clostripain.

**FIG. 4.** Polyacrylamide gel electrophoresis of clostripain digests in the system of Laemmli. The incubation solution contained 11.4 mM Tris-HCl, pH 7.2, 22.8 mM KCl, 50 mM sodium phosphate, pH 7.5, 28.6 μM synthetase, and 4.8 μM clostripain, 0.6 mM dithiothreitol, and 0.24 mM CaCl₂. Incubation was performed at 24 °C. Small samples were removed at the times indicated, in order to characterize proteolytic intermediates, clostripain digests were analyzed by the sodium dodecyl sulfate-polyacrylamide gel electrophoresis method of Schagger and von Jagow (21). The results of this analysis are shown in Fig. 5. Comparison of lanes 14–17 (wild-type) with lanes 1–5 (W76F) permits visualization of the preferential digestion of the β-subunit of W76F added to earlier. Several Coomassie Blue-positive bands were detected, as might have been expected. However, with W76F, two bands, one superimposable on one of the clostripain bands (about 40,000) and the other a more clearly pronounced species of about 31,000, were detected. These bands could also be seen in wild-type, although the 31,000 band was noticeably less intense. An unstained gel was subjected to transblotting and staining, as described under Methods. The band corresponding to the remaining β-subunit gave the expected hexapeptide amino-terminal sequence of H₂N-Met-Asn-Leu-His-Glu-Tyr-Oh (5). Sequencing of the band corresponding to the α-subunit also gave a result that was consistent with the NH₂-terminal six-amino acid sequence expected of this subunit, H₂N-Ser-Ile-Leu-Ile-Asp-Lys-Oh (see below). It would seem from this analysis that the α-subunit remains intact, while most of the β-subunit has been cleaved. Sequencing of the 40,000 band gave the following results for the second through sixth cycle in the analysis, in picomoles: Gly (4.5), Leu (5.2), Pro (3.5), Ala (5.6), and Pro (3.3). No other amino acids appeared in significant amounts. This sequence corresponds to residues 16–20 of the β-subunit. The amino acid cleaved in the first cycle could not be identified, but should have been Tyr which has been determined to be residue 15 (5). Thus, the presence of background polypeptide, probably one of the chains of clostripain, apparently did not interfere with the sequence determination. It is possible that the NH₂-terminus of this polypeptide was blocked. Analysis of the band corresponding to the 31,000 component gave two simultaneous sequences, one corresponding to the amino terminus of the α-subunit, undoubtedly due to tailing of the latter 29,000 species into the 31,000 species in the gel, and the other to H₂N-Leu-Val-Thr-Tyr-Gln-Thr-Oh, which represents residues 81–86 of the β-subunit (5). The α-subunit appeared to transfer much more efficiently to the Immobilon than the 31,000 species, as indicated by the following results of six sequencing cycles, in picomoles: cycle 1, indistinguishable amino acid plus Leu (6.1); cycle 2, Ile (25.0) plus Val (5.1); cycle 3, Leu (25.1) plus Thr (3.2); cycle 4, Ile (24.7) plus Tyr (2.7); cycle 5, Asp (3.7) plus Glu (2.8); and cycle 6, Lys (16.6) plus Thr (2.8). The relatively low transfer yields of polypeptides derived from the digestion products of W76F were included for comparison.
Previous findings of this laboratory indicated that the 3 tryptophan residues of E. coli succinyl-CoA synthetase, all of which reside in the β-subunit, are not required for enzymatic activity and can be replaced by phenylalanines. By fluorescence measurements, 2 of these residues, Trp^W76^ and Trp^W76^, appear to be responsive to the binding of CoA to enzyme (7). The mutant enzymes W76F and W43,76,248F, both of which contain Phe in place of Trp^W76^, were just as sensitive to trypsin in their dephosphorylated states as wild-type enzyme and other tryptophan mutants, but also displayed increased sensitivity to the protease clostripain. Sensitivity to clostripain, a protease that preferentially cleaves peptide bonds at arginine residues, appeared to be independent of phosphorylation state, with regard to both mutant and wild-type enzymes. In addition, the substrate ADP protected the enzyme forms against clostripain but not trypsin. It would appear that trypsin and clostripain represent probes for different sites on the enzyme. The initial cleavage event of clostripain digestion appears to be associated with the ADP-binding site. Trypsin, on the other hand, appears to attack the protein at sites that are uncovered by dephosphorylation with ADP of the enzyme (16).

The use of clostripain as a protease probe of succinyl-CoA synthetase has a distinct advantage over the use of trypsin. The fact that there are 22 unique arginine residues, compared with 46 unique lysine residues, in the enzyme (5) has reduced the number of cleavage sites, since clostripain cleaves preferentially at arginine residues. The use of clostripain also permitted the visualization of intermediate products of proteolysis through the use of the staining procedure of Kratzen et al. (21). It is interesting that, by the procedure of Laemmii (19) (see discussion of Fig. 3), these products were either invisible or poorly defined. In the latter procedure, Coomassie Brilliant Blue R is the staining agent, whereas, in the procedure of Kratzen et al., Coomassie Brilliant Blue G is used.

Detection of identifiable proteolytic products of the β-subunit led to the construction of the double mutants W76F, R14K and W76F,R80K. The resistance of the latter to proteolytic cleavage by clostripain, as well as to loss of enzymatic activity (in contrast to W76F,R14K), coupled with the protective effect of ADP and ATP against inactivation by clostripain, is consistent with the proximity of Arg^W76^ to the ADP(ATP)-binding site. Whether Arg^W76^ serves as a cationic site for ADP or ATP binding is not ruled out by the fact that Lys replacement at this site does not alter enzymatic activity. Under the conditions of the enzyme assay, the lysyl side chain would also be expected to be positively charged. Further experimentation involving site-directed mutagenesis to introduce uncharged or negatively charged side chains at position 80 should serve to settle the matter.

The fact that wild-type succinyl-CoA synthetase, while labile but somewhat less susceptible to clostripain, is also protected against inactivation by this protease by ATP and ADP would appear to indicate that a subtle conformational change has been induced by the Trp to Phe mutation at position 76. This notion is supported by the observation that the CD spectrum of the mutant is superimposable on that of wild-type enzyme (22). Thus, it would appear that the secondary structure of the enzyme is not affected significantly by replacement of Trp^W76^ and that exposure of the peptide bond at Arg^W76^ involves a subtle conformational change.

**DISCUSSION**

The amounts of succinyl-CoA synthetase and clostripain and the conditions employed were as described in the legend of Fig. 1. Wild-type enzyme (○), W76F (△), W76F,R14K (△), and W76F,R80K (●) are shown.

**Fig. 6. Digestion of W76F,R14K and W76F,R80K with clostripain.** The CD spectra of Wild-type Enzyme and W76F—The corrected spectrum of W76F between 180 and 250 nm was superimposable on that of wild-type enzyme (22). Thus, it would appear that the secondary structure of the enzyme is not affected significantly by replacement of Trp^W76^ and that exposure of the peptide bond at Arg^W76^ involves a subtle conformational change.
**Tryptophan Mutants of Succinyl-CoA Synthetase**

Acknowledgment—Peptide sequencing and the synthesis of oligonucleotides were performed by Peggy Rifleman in the Biopolymer Sequencing and Synthesis Facility.

**REFERENCES**