Fluorescence studies and consequences of amino acid substitutions with Escherichia coli adenylosuccinate synthetase

Chandrasen Soans
Iowa State University

Follow this and additional works at: https://lib.dr.iastate.edu/rtd

Part of the Biochemistry Commons, Biophysics Commons, and the Molecular Biology Commons

Recommended Citation
Soans, Chandrasen, "Fluorescence studies and consequences of amino acid substitutions with Escherichia coli adenylosuccinate synthetase" (1992). Retrospective Theses and Dissertations. 9953.
https://lib.dr.iastate.edu/rtd/9953
INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.
Fluorescence studies and consequences of amino acid substitutions with *Escherichia coli* adenylosuccinate synthetase

Soans, Chandrasen, Ph.D.

Iowa State University, 1992
Fluorescence studies and consequences of amino acid substitutions
with *Escherichia coli* adenylosuccinate synthetase

by

Chandrasen Soans

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfilment of the
Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Department: Biochemistry and Biophysics
Major: Biochemistry

Approved:

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

For the Major Department

Signature was redacted for privacy.

For the Graduate College

Iowa State University
Ames, Iowa

1992
Dedicated to the memory of:

my grandparents, Ruth and David Wasker

my grandaunt, Dr. Esther Solomon.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ABBREVIATIONS</strong></td>
<td>v</td>
</tr>
<tr>
<td><strong>GENERAL INTRODUCTION</strong></td>
<td>1</td>
</tr>
<tr>
<td><strong>SECTION I: STUDIES OF LIGAND BINDING TO <em>ESCHERICHIA COLI</em></strong></td>
<td></td>
</tr>
<tr>
<td><strong>ADENYLOSUCCINATE SYNTHETASE</strong></td>
<td>7</td>
</tr>
<tr>
<td><strong>ABSTRACT</strong></td>
<td>9</td>
</tr>
<tr>
<td><strong>INTRODUCTION</strong></td>
<td>10</td>
</tr>
<tr>
<td><strong>EXPERIMENTAL PROCEDURES</strong></td>
<td>12</td>
</tr>
<tr>
<td><strong>RESULTS</strong></td>
<td>16</td>
</tr>
<tr>
<td><strong>DISCUSSION</strong></td>
<td>26</td>
</tr>
<tr>
<td><strong>REFERENCES</strong></td>
<td>31</td>
</tr>
<tr>
<td><strong>SECTION II: FLUORESCENCE RESONANCE ENERGY TRANSFER STUDIES</strong></td>
<td></td>
</tr>
<tr>
<td><strong>AND SITE-DIRECTED MUTAGENESIS WITH <em>ESCHERICHIA COLI</em> ADENYLOSUCCINATE SYNTHETASE</strong></td>
<td>33</td>
</tr>
<tr>
<td><strong>ABSTRACT</strong></td>
<td>34</td>
</tr>
<tr>
<td><strong>INTRODUCTION</strong></td>
<td>36</td>
</tr>
<tr>
<td><strong>EXPERIMENTAL PROCEDURES</strong></td>
<td>41</td>
</tr>
<tr>
<td><strong>THEORY</strong></td>
<td>47</td>
</tr>
</tbody>
</table>
iv

RESULTS 49
DISCUSSION 60
REFERENCES 65

GENERAL SUMMARY 69
LITERATURE CITED 70
ACKNOWLEDGEMENTS 72
### ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMP</td>
<td>adenosine 5'-monophosphate</td>
</tr>
<tr>
<td>AMPS</td>
<td>adenylosuccinate</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine 5'-triphosphate</td>
</tr>
<tr>
<td>DTNB</td>
<td>5, 5'-dithiobis(2-nitrobenzoic acid)</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>FMP</td>
<td>formycin 5'-monophosphate</td>
</tr>
<tr>
<td>GDP</td>
<td>guanosine 5'-monophosphate</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine 5'-triphosphate</td>
</tr>
<tr>
<td>Hepes</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>IMP</td>
<td>inosine 5'-monophosphate</td>
</tr>
<tr>
<td>1,5-I-AEDANS</td>
<td>N-(iodoacetylaminoethyl)-5-naphthylamine-1-sulfonate</td>
</tr>
<tr>
<td>KP&lt;sub&gt;i&lt;/sub&gt;</td>
<td>potassium phosphate</td>
</tr>
<tr>
<td>NBS</td>
<td>N-bromosuccinimide</td>
</tr>
<tr>
<td>NEM</td>
<td>N-ethylmaleimide</td>
</tr>
<tr>
<td>TMRM</td>
<td>tetramethylrhodamine maleimide</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)aminomethane</td>
</tr>
</tbody>
</table>
GENERAL INTRODUCTION

Adenylosuccinate synthetase catalyzes the following reaction:

\[
\text{IMP + GTP + L-aspartate } \xrightarrow{\text{Mg}^{++}} \text{Adenylosuccinate + GDP + Pi}
\]

Adenylosuccinate is then converted to AMP by adenylosuccinate lyase. The reaction catalyzed by adenylosuccinate synthetase represents the first committed step in the de novo synthesis of AMP (1). GTP-binding proteins with an intrinsic GTPase activity usually play important regulatory roles in many cellular processes like housekeeping pathways (2), signalling across membranes (3, 4), protein synthesis in ribosomes (5), etc. Adenylosuccinate synthetase is a GTP-hydrolyzing protein and is extremely important for nucleotide biosynthesis and in the cycling of purine nucleotides and this is borne out especially in neoplastic tissues which have been found to contain an elevated level of the enzyme (6). The appropriate ATP/GTP ratio in the cell is carefully maintained because GTP is essential for adenylate synthesis and ATP for guanylate synthesis. Adenylosuccinate synthetase is regulated by feedback inhibition exerted by its reaction products (7-10).

The enzyme is widely distributed in nature, being found in microorganisms, plants and animals (1). The enzyme is present in the cell in low concentrations leading to difficulties in purifying the enzyme to homogeneity. The enzyme can be obtained in larger quantities from mammalian sources but is still hard to study due to its limited solubility. The
enzyme from *Escherichia coli* which is encoded by the pur A gene, was
cloned, sequenced and overexpressed, and this has made it easier to perform
physical studies on the enzyme (11). The enzyme has also been purified from
*Dictyostelium discoideum* and this cDNA sequence has been reported recently
(12). Mouse muscle adenylosuccinate synthetase has also been cloned and
expressed in COS cells recently (13).

At least two isozymes have been found in vertebrate tissues which can
be distinguished by their isoelectric points (1, 14). The basic form
(pI=8.9) has been observed predominantly in skeletal muscle tissue. The
acidic form (pI=5.9) of the protein is found in non-muscle tissue. They
probably have different roles (15, 16). The acidic isozyme seems to be
involved mainly in the *de novo* synthesis of AMP and the basic isozyme has
been suggested to function mainly in the purine nucleotide cycle (14). The
reported cDNA sequences from *E. coli* and *D. discoideum* have been shown to
encode a protein with kinetic properties similar to the acidic isozyme of
vertebrate tissues (e.g. low $K_m$ for IMP and high $K_m$ for aspartic acid)
(12). The amino acid sequences for the enzymes from all three sources show
extensive homology (13). Sequence analysis of adenylosuccinate synthetase
from all three sources reveal functional domains typical of GTP-binding
proteins like elongation factor 2, and H-ras. A phosphoryl-binding domain
Gly-X-X-X-Gly-Lys and a GTP hydrolysis domain Asp-X-X-Gly as well as the
guanine specificity binding site Asn-Lys-X-Asp consensus sequence is
present in the cDNA sequence of all three proteins (13). Previous studies
on adenylosuccinate synthetase involving chemical modification and site-
directed mutagenesis have shown that Lys$^{140}$ is involved in GTP-binding and
Arg\textsuperscript{147} is involved in the binding of both GTP and IMP (16,17). These residues are conserved in the synthetases from both \textit{D. discoideum} (Lys\textsuperscript{143}, Arg\textsuperscript{150}) and mouse muscle (Lys\textsuperscript{174}, Arg\textsuperscript{181}) (12, 13).

Adenylosuccinate synthetase has complex control mechanisms and various inhibitors have been used to clarify them. AMP, GDP, GMP and adenylosuccinate are feedback inhibitors of the enzyme. The aspartate analog hadacidin, and allopurinol ribonucleotide (4-hydroxypyrazolo[3,4-d]pyrimidine-ribose-5'-P) are naturally occurring antibiotics and have been shown to have antitumour, antigrowth activity in \textit{D. discoideum} and \textit{Trypanosoma cruzi} respectively (1).

The synthetase from \textit{E. coli} is enzymatically active as a dimer with a subunit molecular mass of 48,000 (1). Numerous studies have been done to elucidate the mechanism of the reaction. In 1956, Lieberman suggested from the results of his \textsuperscript{18}O incorporation studies that 6-phosphoryl-IMP might be an intermediate in the reaction (18). Fromm (1958) came to the same conclusion from equilibrium-exchange studies of the reaction (19). On the other hand, Hartman and Buchanan (1958) suggested that the interaction of all three substrates occurs at the enzyme surface in a concerted fashion without any new covalently-linked compounds being formed as obligatory intermediates (20, 21). They based this proposal on the observation that no partial reactions are observed even with arsenate replacing phosphate in the reaction i.e., all three products are required to obtain reversal of the reaction. Rudolph (1969) however, noted that the protein might have to be in the proper conformation for GDP to bind and for the reaction to occur in the reverse direction and hence the absence of partial reactions did not
mean that covalent intermediates were not formed (22). Isotope scrambling experiments by Bass and Fromm have offered more proof of a 6-phosphoryl-IMP intermediate in the adenylosuccinate synthetase reaction (23). During X-ray crystallography studies it was observed that GTP, GDP and IMP cause cracking of the adenylosuccinate crystal presumably by causing some sort of a conformational change in the protein, while soaks with aspartate caused no apparent change in the crystal, consistent with the hypothesis that IMP and GTP interact prior to any interaction with aspartate (24). Chemical modification studies using phenylglyoxal by Dong and Fromm showed that the enzyme activity could be protected only if both IMP and GTP were present. Aspartate offered no protection against inactivation by phenylglyoxal (25). These observations offer more evidence to show that IMP and aspartate bind to the enzyme at topologically different locations. This report also shows that the enzyme exists in different conformational states when GTP and GDP are bound as seen from the absence of quenching of the intrinsic fluorescence of the enzyme by GDP but not by GTP, and also from the different degrees of fluorescence enhancement seen with the enzyme and TbGTP and the enzyme and TbGDP. The formation of dead-end complexes like enzyme-TbGTP-adenylosuccinate-aspartate also goes to show that IMP and aspartate have distinct binding sites on the enzyme. A third mechanism proposed that aspartate attacks the C6 of IMP to form an intermediate which is then phosphorylated by GTP to form the products (26).

Most of the earlier investigations on adenylosuccinate synthetase focussed on shedding light on the reaction mechanism using a variety of techniques like kinetic studies, isotope exchange, nuclear magnetic
resonance, etc. Using IMP analogs, Hampton and Chu were the first to show that IMP binds to the enzyme as a dianion and that the phosphoryl group of IMP is important for both binding and catalysis (27). Recently, however, much more has been done to study the enzyme-substrate interaction. From the amino acid sequence of the E. coli enzyme, which was deduced from the nucleotide sequence, it is known that there are 4 Cys residues, 22 Lys residues and 28 Arg residues present in the enzyme. We now know from the chemical modification and site-directed mutagenesis work of Dong et al. that one of the 22 Lys residues (Lys$^{140}$) is important for enzyme activity and is probably involved in GTP binding, and one of the 28 Arg residues (Arg$^{147}$) is important for catalysis while none of the Cys residues are important for enzyme activity or substrate binding under physiological conditions (16, 25, 28). The N-terminal glycine rich sequence GDEGKGK in the enzyme which is homologous to the conserved sequence GXXXXGK found in other guanine nucleotide-binding proteins was also shown to be extremely important for enzyme catalysis though not so important for substrate binding (17).

Section I of this dissertation describes an attempt to gain insight into the microenvironment surrounding certain amino acid residues of the enzyme using fluorescent ligands that covalently bind to the enzyme. Information about the conformational changes that the enzyme goes through on complexing with active site binding ligands was acquired using either the intrinsic fluorescence of the enzyme or fluorescent ligands. Dissociation constants of active site binding ligands that quench the intrinsic fluorescence of the enzyme are reported.
Section II describes distance measurements from Cys\(^{291}\) on the enzyme to the IMP and GTP binding sites using fluorescence resonance energy transfer. These experiments were performed in order to gain some knowledge about the physical structure of the enzyme. The results of the site-directed mutagenesis experiments involving Cys\(^{344}\), Trp\(^{309}\) and Trp\(^{378}\) are also reported.

**Explanation of dissertation format:** The dissertation is written in the alternate format. The author has played a primary role in the research and writing of this dissertation. The first section and a part of the second section have been published in scholarly journals. A General Summary follows the second paper and a list of references used in the General Introduction and General Summary follows the General Summary.
SECTION I: STUDIES OF LIGAND BINDING TO *ESCHERICHIA COLI* ADENYLOSUCCINATE SYNTHETASE.
Studies of Ligand Binding to *Escherichia coli*

Adenylosuccinate Synthetase*

Chandrasen Soans and Herbert J. Fromm

Department of Biochemistry and Biophysics,

Iowa State University, Ames, Iowa 50011

* This research was supported in part by Research Grant NS10546 from the National Institutes of Health, United States Public Health Service, and Grant DMB-8902211 from the National Science Foundation. This is Journal Paper J-14172, Project 2575, of the Iowa Agriculture and Home Economics Experiment Station, Ames, IA.
ABSTRACT

Dissociation constants of *Escherichia coli* adenylosuccinate synthetase with IMP, GTP, adenylosuccinate, and AMP (a competitive inhibitor for IMP) were determined by measuring the extent of quenching of the intrinsic tryptophan fluorescence of the enzyme. The enzyme has one binding site for each of these ligands. Aspartate and GDP did not quench to any great extent, and their dissociation constants could not be determined. These ligand binding studies were generally supportive of the kinetic mechanism proposed earlier for the enzyme. Cys\(^{291}\) was modified with the fluorescent chromophores N-(iodoacetylaminoethyl)-5-naphthylamine-1-sulfonate and tetramethylrhodamine maleimide in order to measure enzyme conformational changes attending ligand binding. The excitation and emission spectra of these fluorophores are not altered by the addition of active site binding ligands. TbGTP and TbGDP were used as native reporter groups, and changes in their fluorescence on complexing with the enzyme and various ligands made it possible to detect conformational changes occurring at the active site. Evidence is presented for abortive complexes of the type: enzyme-TbGTP- adenylsuccinate and enzyme-TbGTP-adenylsuccinate-aspartate. These results suggest that the IMP and aspartate binding sites are spatially separated.
INTRODUCTION

Adenylosuccinate synthetase [IMP:L-aspartate ligase (GDP-forming), EC 6.3.4.4] represents the first committed step in the conversion of IMP to AMP and plays a crucial role in the purine nucleotide cycle (1). Initial rate experiments (2) indicate that the reaction mechanism is Random Ter Ter; however, isotope exchange studies at chemical equilibrium (3) imply that the substrate addition involves a preferred pathway with aspartate adding to the enzyme after the addition of the nucleotide substrates. There is evidence from isotope scrambling experiments that 6-phosphoryl-IMP may well be an intermediate in the adenylosuccinate synthetase reaction (4) as suggested earlier by Lieberman (5) and subsequently Fromm (6).

The adenylosuccinate synthetase gene was recently cloned, and the enzyme overexpressed in Escherichia coli (7). Chemical modification experiments and studies involving site-directed mutagenesis (8) showed that Cys^291 does not seem to be involved in either catalysis or substrate binding, and therefore, has the potential to act as an anchor for "reporter groups," which could be used to monitor enzyme conformational changes. Labeling of Cys^291 with sulfhydryl specific fluorescent reagents offers a convenient means of studying the microenvironment around this residue as well as the relationship of this residue relative to the substrate and product binding sites without significantly affecting enzyme activity. In this study, we report the modification of Cys^291 with two sulfhydryl specific fluorescent reagents, 1,5-I-AEDANS (9,10) and TMRM (11-13), in order to evaluate conformational changes that accompany ligand binding. This point was also pursued with TbGTP and TbGDP. Virtually nothing is
known regarding conformational changes that attend ligand binding to the enzyme. In addition, we report the dissociation constants of adenylosuccinate synthetase with IMP, GTP, adenylosuccinate, and AMP. These parameters are not available in the literature. Finally, results from the fluorescence studies seem to support the mechanism that was alluded to in isotope scrambling experiments (4).
EXPERIMENTAL PROCEDURES

Materials: IMP, GTP, GDP, AMP, FMP, L-aspartate, adenylosuccinate, and 1,5-I-AEDANS were obtained from Sigma Chemical Co. TMRM was from Molecular Probes. Terbium (III) chloride hexahydrate and quinine sulfate monohydrate were from Aldrich. Distilled, deionized water was used exclusively. All other chemicals were obtained from commercial sources and were of the highest quality available.

Preparation of adenylosuccinate synthetase: Adenylosuccinate synthetase was prepared from E. coli as described previously (7). The purified enzyme was dialyzed against 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA at 4°C. The concentration of the purified enzyme was measured spectrophotometrically by using the Bio-Rad protein assay with crystalline bovine serum albumin as the standard. The concentration of the enzyme refers to the enzyme monomer. Enzyme activity assays were performed at 25°C by using the rate of appearance of adenylosuccinate as determined from the absorbance at 280 nm on a Cary 118 spectrophotometer set to 0.1 absorbance units full-scale as described previously (14).

Modification of adenylosuccinate synthetase with 1,5-I-AEDANS: A total of 300 μl of 13.1 mg/ml purified adenylosuccinate synthetase in 50 mM potassium phosphate (pH 7.0) was incubated in the dark with 100 μl of 1,5-I-AEDANS (5.8 mg/ml) for 15 min on ice. The enzyme was dialyzed overnight against 50 mM potassium phosphate to remove excess modifying reagent.

Modification of adenylosuccinate synthetase with TMRM: A total of 13.8 mg/ml purified enzyme in 1 ml of 50 mM phosphate buffer (pH 7.0) was incubated with stirring for 18 h in the dark at 3°C with a 3 M excess of
The modified enzyme was washed several times with 50 mM phosphate buffer by using an Amicon Centricon 30 microconcentrator to remove excess maleimide.

**Fluorescence measurements:** Hepes (25 mM, pH 7.7) was used to buffer the samples to be used in the fluorescence experiments. Fluorescence measurements, using 1-cm path length quartz cell thermostated at 20°C, were carried out in a SLM 80000 spectrofluorometer equipped with computer-controlled data accumulation and processing capabilities. Fluorescence emission spectra were corrected for fluorescence of ligands, enzyme, and reagents when necessary. All calculations were corrected for dilution of solutions. All reaction mixtures were allowed to incubate at 20°C to reach equilibrium before the fluorescence measurements were made. Enzyme solutions were excited at 280-300 nm, and the intensity of protein fluorescence was measured at 333 nm. If necessary, the observed fluorescence intensity was corrected for the absorption of incident light by nucleotides or by substrate. The slit widths on the excitation and emission monochromators were 2 mm. Quantum yields were determined relative to a value of 0.55 for quinine sulfate in 0.1 N H₂SO₄ (15) using an exciting wavelength of 350 nm. The comparative relationship of Parker and Rees (16) was used:

\[
\frac{Q_2}{Q_1} = \frac{(F_2 \cdot A_1)}{(F_1 \cdot A_2)}. \tag{1}
\]

Q₂ is the quantum yield of the unknown, F₂ the area of the corrected emission spectrum, and A₂ the absorbance at the exciting wavelength. Q₁, F₁, and A₁ are the corresponding values for the standard quinine sulfate.
The computer paired with the spectrofluorometer was used to calculate the areas of corrected emission spectra. Absorbance was measured on a Cary 118 spectrophotometer. Samples used for fluorescence measurements usually had an absorbance below 0.05 at the exciting wavelength to avoid inner-filter quenching. Blanks were used to correct for baseline variations as a function of wavelength during both absorption and fluorescence measurements. Tb(III) solutions were prepared dilute and at pH 7.5 to avoid precipitation of Tb(III) as Tb(OH)₃. A 3:1 stoichiometry ratio of Tb:GTP or Tb:GDP was maintained at which 99.7% GTP or GDP was in the form TbGTP or TbGDP.

**Calculation of dissociation constants from fluorescence measurements:** The following relationship exists when an enzyme (E) and the related enzyme-ligand (EL) complex both fluoresce at a single wavelength,

\[ F = \alpha \cdot E + \beta \cdot EL, \]  \[ [2] \]

and when \( L_t \gg E_t \), the following equation is valid,

\[ E_t - F = \frac{(\alpha-\beta) \cdot E_t \cdot L_t}{(K_1 + L_t)} \]  \[ [3] \]

where \( F \) is the observed fluorescence, \( EL \) is the concentration of enzyme-ligand complex, \( L_t \) is the total ligand concentration, \( E_t \) is the total enzyme concentration, \( K_1 \) represents the dissociation constant of the enzyme-ligand complex, and \( \alpha \) and \( \beta \) are the molar fluorescent emittances of enzyme and enzyme-ligand, respectively (17).
In the determination of the dissociation constants of the enzyme-ligand complexes, the following equation was used:

$$\frac{1}{(\alpha E_t - F)} = \frac{1}{(\alpha - \beta)E_t} + \frac{K_I}{(\alpha - \beta)E_t} L_t.$$  \[4\]

Dissociation constants were calculated from computer simulations by using ENZFITTER, a nonlinear regression data-fitting program (18).
RESULTS

In order to determine the dissociation constants of IMP, GTP, GDP, AMP, aspartate, and adenylosuccinate, advantage was taken of the fact that none of these compounds exhibits fluorescence. The formation of adenylosuccinate synthetase-ligand complexes was followed by observing the quenching of enzyme fluorescence by the ligand. The results from the binding studies are shown in Table I and are compared to the Michaelis constants in the literature (1). Unfortunately, there is no direct correlation between $K_d$ and $K_m$ values, and these data represent the only attempt to record $K_d$ measurements of the E. coli enzyme. Figure 1 shows a plot of the quenching of enzyme fluorescence by adenylosuccinate. The inset describes a plot of the binding data, which yielded the $K_d$. Similar plots were obtained for IMP, GTP, and AMP (data not shown). The amount of quenching at saturating concentrations of the ligands is also shown in Table I. AMP, a competitive inhibitor of IMP, which binds the enzyme less tightly than IMP, actually quenches about 7% more than IMP. This alteration, which we believe is significant, indicates that there are some differences in the manner of binding of the two ligands to the enzyme. The dissociation constants for aspartate and GDP could not be determined because they did not cause significant quenching of enzyme fluorescence which is essential when calculating dissociation constants by this method. This methodology can be extended to determining $K_d$ values for ternary complexes of enzyme and two substrates; however, because the kinetic mechanism is very probably steady-state random (3), binding parameters obtained from such experiments would provide little information on the details of the mechanism.
TABLE I: Comparison of Kinetic Constants with Dissociation Constants as Determined from Fluorescence Measurements

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Kinetic constants (K_m or K_i) (μM)</th>
<th>Dissociation constants from fluorescence measurements (K_d) (μM)</th>
<th>% Quenching at saturating concentrations</th>
<th>Number of ligand binding sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>IMP</td>
<td>20</td>
<td>37.7 ± 1.9</td>
<td>21</td>
<td>1</td>
</tr>
<tr>
<td>GTP</td>
<td>10</td>
<td>23.7 ± 0.6</td>
<td>9.0</td>
<td>1</td>
</tr>
<tr>
<td>AMP</td>
<td>95</td>
<td>148 ± 5</td>
<td>28</td>
<td>1</td>
</tr>
<tr>
<td>AMPS</td>
<td>5.0</td>
<td>1.80 ± 0.04</td>
<td>27</td>
<td>1</td>
</tr>
<tr>
<td>GDP</td>
<td>12</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Aspartate</td>
<td>350</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*aFrom Ref. (1).*
Figure 1. Plot of the quenching of enzyme fluorescence with adenylosuccinate. Plot of the decrease in protein fluorescence intensity at 333 nm versus the (micromolar) concentration of total adenylosuccinate added. The solution was excited at 300 nm and contained 25 mM Hepes buffer, pH 7.7. (Inset) Plot of the reciprocal of $\alpha[E]_t - F$ versus the reciprocal of the concentration of total adenylosuccinate.
The number of ligand binding sites associated with adenylosuccinate synthetase: A Scatchard plot was used to determine the number of ligand binding sites on the enzyme. When the binding sites of ligand on the enzyme are identical and noninteracting, the Scatchard plot has the form

\[ \frac{v}{L} = \frac{n}{K} - \frac{v}{K}, \tag{5} \]

where \( v = EL/E_t \), \( n \) is the number of binding sites, \( L \) is the final concentration of free ligand, and \( K \) is the dissociation constant. \( EL/E_t \) is calculated from the equation

\[ EL = \frac{\alpha E_t - F}{\alpha - \beta}, \tag{6} \]

where \( \alpha \) and \( \beta \) are the molar emittances of the enzyme and of the enzyme-ligand complex, respectively (17).

Figure 2 shows a Scatchard plot for adenylosuccinate. Similar plots with other ligands showed that the enzyme has one binding site for each of the ligands. These findings are fully consistent with results from initial rate experiments for adenylosuccinate synthetase (1).

The effect of ligands on the fluorescence properties of the chemically modified enzyme: In order to insert a single fluorescent probe into the enzyme, labeling was carried out with an excess of either 1,5-I-AEDANS or TMRM. Under these conditions, 0.98 mol of AEDANS and 0.92 mol of TMRM were incorporated per mol of enzyme. We know from previous experiments that these fluorophores bind to Cys\textsuperscript{291} (8). Bound AEDANS or TMRM was determined
Figure 2. Scatchard plot of the titration of the enzyme (0.22 μM) with adenylsuccinate. The concentration of the enzyme-ligand complex was calculated from Eq. [6] as indicated in the text. The concentration of the free nucleotide was obtained by subtracting the concentration of the enzyme-ligand from the total nucleotide concentration. The number of the binding sites was obtained from the x-intercept to be 0.96 sites per subunit of enzyme.
by assuming that $\varepsilon^{337} = 6100 \text{ M}^{-1} \text{ cm}^{-1}$ (9) and that $\varepsilon^{555} = 5000 \text{ M}^{-1} \text{ cm}^{-1}$ (13) for AEDANS and TMRM, respectively. These preparations of the fluorescent labeled enzyme were used in the experiments described below. Upon addition of adenylosuccinate synthetase to a solution of 1,5-I-AEDANS, an enhancement in the fluorescence of the latter was seen (data not shown). The emission maxima were also shifted slightly to shorter wavelengths, suggesting that the fluorophore is binding to a hydrophobic part of the enzyme molecule (9). Adenylosuccinate synthetase modified with 1,5-I-AEDANS when excited at 337 nm fluoresced at 480 nm. Addition of MgGTP, IMP, aspartate, or adenylosuccinate separately did not cause significant changes in the emission spectrum. Addition of MgGTP and IMP, or aspartate, or IMP and MgGTP, or aspartate did not cause any changes in the enzyme-AEDANS fluorescence spectrum. Similar results were obtained when adenylosuccinate synthetase modified with TMRM was treated as above. It is clear from these studies that ligand binding at the active site does not alter the environment around the fluorescent label.

Factors affecting properties of the fluorescent ligands: FMP is a fluorescent structural analog of AMP (19, 20) and has been used effectively as a substitute for AMP in a variety of enzymatic reactions (21). FMP is a competitive inhibitor relative to IMP (data not shown). The fluorescent properties of FMP at neutral pH and 20°C have been characterised. The effects of various ligands on the fluorescence of the FMP-enzyme complex were examined. No alteration in fluorescence was seen with active site binding ligands such as MgGTP, MgGDP, aspartate, and adenylosuccinate.

A number of complex and important biological processes involve GTP.
Mammalian succinyl-CoA synthetase, phosphoenolpyruvate carboxykinase, and guanylate cyclase are some examples of enzymes that are specific for GTP. The mechanisms of these biological reactions have been studied using chromophoric or fluorescent analogs of GTP. Adenylosuccinate synthetase requires Mg** for activity. The metal does not bind to the enzyme, but instead it serves to chelate GTP. It is known that Tb(III) and Eu(III) can exhibit considerably enhanced fluorescence when replacing Ca(II) in calcium-dependent proteins or when bound to guanosine nucleotides (22). We found that TbGTP binds to adenylosuccinate synthetase and can replace MgGTP; i.e., it is competitive relative to MgGTP; however, the system is not catalytically active. The TbGTP-enzyme complex possesses characteristic fluorescence properties and is therefore a suitable fluorescent probe for the study of the microenvironment of the substrate site on adenylosuccinate synthetase and thus has the potential to yield specific information about the structure of the enzyme. It can also be used to monitor the conformational transitions the enzyme undergoes on binding to substrates or products. TbCl$_3$ in Heps buffer (pH 7.7) does not show any appreciable fluorescence in the visible spectral region when excited at 295 nm, but when complexed with GTP, it has two emission peaks, one at 488 nm and the other at 544 nm. Kumar and Chatterji (23) have shown that Tb(III) binds to GTP mainly by tridentate coordination, and they have calculated a dissociation constant of 0.2 μM for TbGTP. TbGTP is a useful fluorescent probe of hydrophobic microenvironments as indicated by the fact that on binding to the enzyme, the fluorescence was enhanced about 1.5-fold (Figure 3). The enhancement is due to the complex formation between TbGTP and
adenylosuccinate synthetase. The enzyme alone did not cause Tb(III) to fluoresce. Addition of IMP enhanced the fluorescence to about 2.0-fold. Subsequent addition of aspartate increased the fluorescence only slightly. On the other hand, addition of aspartate to the TbGTP-enzyme complex leads to a 1.8-fold enhancement of fluorescence as shown in Table II, but subsequent addition of IMP did not significantly enhance additional fluorescence. As described in Table II, addition of adenylosuccinate to the TbGTP-enzyme complex caused a 2.9-fold enhancement of fluorescence. Subsequent addition of aspartate caused the fluorescence to increase 3.7-fold over the original fluorescence (Table II).

Addition of enzyme to a solution of TbGDP caused the fluorescence to double as depicted in Table II. Addition of IMP or aspartate caused additional fluorescence enhancement (Table II). Once any of the above ligands had been added to the TbGDP-enzyme complex, subsequent addition of the other ligand caused only a small increase in fluorescence. On the other hand, as shown in Table II, addition of adenylosuccinate to the TbGDP-enzyme complex caused a fourfold increase of the TbGDP fluorescence. These results suggest that GDP alone does indeed bind to adenylosuccinate synthetase at least as TbGDP. It is certainly possible that MgGDP does not bind to the enzyme; however, it is more likely that in the absence of other substrates, the complex of enzyme and MgGDP does not cause a conformational change in the enzyme that is reflected by an alteration in intrinsic fluorescence. This conclusion is in harmony with kinetic data that suggest that GDP binds tightly to adenylosuccinate synthetase (1,2).
Figure 3. Corrected fluorescence emission spectra of 100 μM Tb and 30 μM GTP with adenylosuccinate synthetase and IMP. (A) TbGTP in 25 mM Hepes buffer, pH 7.7; (B) after the addition of 0.5 μM adenylosuccinate synthetase to A; (C) after the addition of 41.6 μM IMP to B. Fluorescence excitation was at 295 nm.
TABLE II: Effect of Adenylosuccinate Synthetase and Various Ligands on the Fluorescence of TbGTP or TbGDP

<table>
<thead>
<tr>
<th>Additions to adenylsuccinate synthetase + Tb-nucleotide</th>
<th>Increase in fluorescence relative to free TbGTP (-fold)</th>
<th>Increase in fluorescence relative to free TbGDP (-fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1.5</td>
<td>1.9</td>
</tr>
<tr>
<td>IMP</td>
<td>1.9</td>
<td>2.4</td>
</tr>
<tr>
<td>IMP + aspartate</td>
<td>2.1</td>
<td>2.6</td>
</tr>
<tr>
<td>None</td>
<td>1.5</td>
<td>2.0</td>
</tr>
<tr>
<td>Aspartate</td>
<td>1.8</td>
<td>2.4</td>
</tr>
<tr>
<td>Aspartate + IMP</td>
<td>1.8</td>
<td>2.5</td>
</tr>
<tr>
<td>None</td>
<td>1.5</td>
<td>2.0</td>
</tr>
<tr>
<td>AMPS</td>
<td>2.9</td>
<td>4.0</td>
</tr>
<tr>
<td>AMPS + aspartate</td>
<td>3.7</td>
<td>Not determined</td>
</tr>
</tbody>
</table>
DISCUSSION

The experiments contained in this report were carried out in an attempt to obtain insights into the mechanism of action of adenylosuccinate synthetase. Initial rate studies suggest that the kinetic mechanism is rapid equilibrium random (2); however, there is reason to believe that the mechanism is in fact steady-state random (3). Results from isotope exchange experiments suggest that there is a preferred pathway of substrate addition to the enzyme; e.g., GTP and IMP add randomly followed by the addition of aspartate (3). We have recently identified two substrate binding regions of the enzyme (24, 25). It was observed from chemical modification and site-directed mutagenesis studies that Lys$^{140}$ is important for GTP binding, whereas Arg$^{147}$ is involved in both GTP and IMP binding. In addition, we have found (unpublished observations) that the phosphate loop near the N-terminus is also involved in nucleotide substrate binding. These findings are generally supportive of the fluorescent data contained in this report.

As shown in Table I, there is no evidence of aspartate binding to adenylosuccinate synthetase in the absence of nucleotide substrates; however, such binding does occur in the presence of either IMP or GTP as can be seen in Table II. Aspartate was not able to prevent chemical modification at the active site amino acid residues Lys$^{140}$ or Arg$^{147}$, nor was the $K_m$ for aspartate significantly altered when these amino acids and those in the phosphate loop were changed by site-directed mutagenesis (24, 25).

It is useful to correlate various parameters obtained by kinetic
experiments with those determined by other methods for adenylosuccinate synthetase. The $K_d$ values reflect the affinity of the enzyme for the substrate in the absence of the two co-substrates, whereas the Michaelis constant in such a system, represents a complex constant for a substrate when the other two substrates are bound to the enzyme, assuming the kinetic mechanism is steady-state random. Unfortunately, the kinetic data in the literature for the E. coli enzyme describe $K_m$ and not $K_d$ values (2). As can be seen in Table I, quenching of intrinsic adenylosuccinate synthetase fluorescence by IMP, GTP, AMP, and adenylosuccinate permitted evaluation of their $K_d$s. The dissociation constants for aspartate and GDP could not be determined because they did not cause significant enhancement or quenching of the enzyme fluorescence, which is essential when using this method. This finding is interesting because GDP is known to bind tightly to the enzyme (2), yet it does not cause a change in intrinsic enzyme fluorescence. This observation leads to the suggestion that the enzyme exists in different conformational states when GTP and GDP are bound.

Quenching of enzyme fluorescence in the presence of substrates as described in Table I suggests the possibility of a direct interaction between tryptophan and the ligand causing quenching of tryptophan fluorescence. On the other hand, these results may be interpreted to mean that there are changes in the enzyme conformation induced by the binding of substrate to the active site that alters the environment near the fluorescing tryptophan residue, thus causing quenching. The tryptophan in question need not be in proximity to the active site.

It is known that the fluorescence characteristics of chromophores are
sensitive to their microenvironment (9). Simply monitoring the intensity of
the fluorescence of an intrinsic or extrinsic probe can give some
information on the immediate surroundings. In general, the fluorescence
intensity increases as the polarity of the environment decreases (9). The
observed increase in fluorescence intensity and small blue shift of the
emission maximum of 1,5-I-AEDANS upon binding to the enzyme can be
explained as the enzyme interacting directly with the chromophore and
binding it in a region of increased hydrophobicity in the environment of
the chromophore. In fact, previous experiments have provided some evidence
indicating that Cys²⁹¹ lies within an enzyme crevice (8).

TbGTP is another useful "environmental probe" because the
fluorescence of the complex is greatly enhanced on binding the enzyme. As
shown in Figure 3 and Table II, the present study revealed that there is a
fluorescence enhancement in the presence of the enzyme, which increases in
the presence of IMP or aspartate (Table II). This suggests that the
microenvironment of the TbGTP becomes more hydrophobic or more rigid upon
binding to the enzyme alone or to a ligand-associated enzyme; in other
words, conformational changes are occurring at the active site. These
changes, however, do not seem to be transmitted to the environment around
Cys²⁹¹ because no significant changes in the fluorescence of either
covalently bound AEDANS or TMRM were observed upon addition of ligands to
the modified enzyme. As can be seen from the data of Table II, no
significant enhancement of fluorescence was observed upon addition of
aspartate to the TbGTP-enzyme-IMP complex or upon addition of IMP to the
TbGTP-enzyme-aspartate complex (Table II), indicating that once either GTP
and IMP or GTP and aspartate are present, the enzyme is "pulled" into the catalytic conformation, and no further significant conformational changes occur on addition of the third substrate, at least as monitored by our system. Interestingly, as shown in Table II, there is a significant enhancement in the fluorescence of the TbGTP-enzyme complex upon the addition of adenylosuccinate, which increases upon the addition of aspartate (Table II). These observations provide evidence for the formation of enzyme-TbGTP-adenylosuccinate and enzyme-TbGTP-adenylosuccinate-aspartate abortive ternary and quarternary complexes, respectively. The fact that adenylosuccinate and aspartate can bind to the enzyme simultaneously supports the suggestion, alluded to from kinetic studies, that the IMP and aspartate sites are spatially separated (1). The fluorescence enhancement observed upon addition of enzyme to TbGDP is greater than that with TbGTP. Differences in the binding or orientation of TbGTP and TbGDP may be due to differences in enzyme conformation in the presence of each of these ligands and are consistent with the quenching results discussed earlier.

There is evidence to show that GTP binds to the enzyme in a syn conformation (M. Roy and H. J. Fromm, unpublished results). How IMP binds to the enzyme is not known. The only difference in the chemical structure of IMP and AMP is the presence of the amino or hydroxyl group at the C-6 of the purine ring. AMP is a competitive inhibitor relative to IMP and presumably binds to the enzyme at the IMP binding site; however, there are measureable differences in the binding of these two nucleotides to adenylosuccinate synthetase as monitored by fluorescence. Thus, the
interactions formed by AMP with the side chains of the amino acid residues on the enzyme are not as strong as the ones formed by IMP. This is reflected in the differences in the binding constants. The maximum quenching of IMP and AMP at saturating levels of the ligands are also different. Interestingly, AMP quenches enzyme fluorescence almost as well as does adenylosuccinate, which has a nitrogen atom at the C-6 position.
REFERENCES


SECTION II: FLUORESCENCE RESONANCE ENERGY TRANSFER STUDIES AND SITE-DIRECTED MUTAGENESIS WITH \textit{ESCHERICHIA COLI} ADENYLOSUCCINATE SYNTHETASE
ABSTRACT

Probes were introduced into the active site of adenylosuccinate synthetase from *Escherichia coli* for fluorescence energy transfer measurements. The distances from fluorescent reporter groups covalently bound to a highly reactive cysteinyl residue (Cys\(^{391}\)) of adenylosuccinate synthetase, to active site binding ligands were determined. Energy transfer from formycin 5'-monophosphate, a competitive inhibitor of IMP, to Cys\(^{291}\), modified with the fluorescent chromophore N-(iodoacetylaminoethyl)-5-naphthylamine-1-sulfonate, indicated that the chromophores are 17 Å apart in the presence or absence of GTP. Analogous experiments using tetramethylrhodamine maleimide to modify Cys\(^{291}\) and TbGTP revealed a distance of 9 Å in the presence or absence of IMP. The fluorescent energy transfer studies support the suggestion that the substrates GTP and IMP add randomly to the enzyme. Transfer of excitation energy also occurred from the protein moiety of the enzyme to the fluorescent label on Cys\(^{291}\) with an efficiency of 34%. It has been shown previously that modification of Cys\(^{344}\) affects the activity of the enzyme. When Cys\(^{344}\) was replaced by either serine or alanine, the mutant enzymes were found to be as active as the wild-type enzyme pointing to the nonessential role of Cys\(^{344}\) in adenylosuccinate synthetase. Site-directed mutagenesis of Trp\(^{309}\) to Phe and Trp\(^{378}\) to Phe were done separately to produce two mutant forms of the enzyme. Comparison of the kinetic properties of the wild-type and those of the mutant enzymes revealed that the \(k_{\text{cat}}\) of the mutants were 28% less than the wild type suggesting that perhaps it was required to maintain the three dimensional structure of the enzyme. There were no significant differences in the Michaelis constants of
the mutant enzymes and the wild type showing that they do not play a role in substrate binding. Some differences were noted in the fluorescence spectra of the mutants indicating differences in the microscopic environments of these amino acids.
INTRODUCTION

Adenylosuccinate synthetase catalyses the formation of adenylosuccinate from aspartate and IMP and converts GTP to GDP in the process (1). The enzyme has been well characterised from different species and tissues and was first purified to homogeneity from Escherichia coli in 1976 (1). The enzyme from E. coli is a homodimer with a subunit molecular mass of 48,000 (2). Most of the research on adenylosuccinate synthetase has been directed towards studying the substrates and products and not much is known about the enzyme structure itself. X-ray diffraction studies and nuclear magnetic resonance spectroscopy (NMR) investigations are now in progress.

Chemical modification experiments on adenylosuccinate synthetase using thiol reagents like 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) or N-ethylmaleimide (NEM) leads to the modification of one cysteine residue per enzyme monomer with the enzyme retaining most of its activity. Under mild denaturing conditions (3.5 M urea), a second cysteine residue can be modified. The derivatization of this residue followed by dialysis results in total loss of enzyme activity. The remaining two cysteines are modified only under stronger denaturing conditions (8 M urea). By using[^14C]NEM to modify the enzyme followed by digestion with trypsin and radiodepeptide isolation, the location of the reactive Cys was identified as Cys^{291} and the cysteine residue that is exposed only on treatment with 3.5 M urea was identified as Cys^{344} (3). It has been previously reported that some eucaryotic adenylosuccinate synthetases have sulfhydryl groups that are essential for enzyme activity (4,5). However, not much is known about the
reactivity of the cysteinyl residues of the procaryotic enzymes. Once the status of the thiol groups in the enzyme were established it would prove useful in future fluorescence, ligand binding, NMR, and X-ray diffraction studies. Hence the site-directed mutagenesis of Cys$^{344}$ to Ala and Ser was performed. The results of the site-directed mutagenesis experiments are reported here and show Cys$^{344}$ is not essential for catalytic activity.

Information about the separation between molecules can be obtained by measuring the transfer of electronic excitation energy between these molecules and this knowledge can be used to study interaction between molecules and structural alterations of macromolecules (6). Structural changes in proteins resulting from their interaction with the environment or with ligands such as substrates can be detected with great sensitivity using fluorescence spectroscopy (7). Fluorescence measurements offer particular advantages over other spectroscopy techniques since they can be used in the study of biological molecules at relatively low concentrations. The intrinsic fluorescence of proteins by tryptophanyl and tyrosyl residues has been used extensively in structure-function studies (8-11). Such structure function studies would be helpful in answering basic questions, such as, how enzymes fold, how they recognize substrates and how they catalyze reactions. Knowledge of the geometric relationship between Cys$^{291}$ and the IMP and GTP binding sites could lead to a better understanding of the catalytic mechanism.

The fact that the modification of Cys$^{291}$ does not affect enzyme activity makes it a valuable site for reporter groups. The modification of Cys$^{291}$ with TMRM (12-14) and 1,5-I-AEDANS (15, 16), two sulfhydryl specific
florescent reagents is reported here. The absorption band of 1,5-I-AEDANS overlaps the emission band of FMP (a fluorescent analog of AMP and a competitive inhibitor to IMP). Thus these two form a Förster donor-acceptor pair. Similarly TMRM has an absorption peak, which overlaps the emission peaks of the TbGTP-enzyme complex at 488 and 544 nm. Therefore, in principle, these two can form a Förster’s donor-acceptor pair provided they are located at suitable distances from each other. Indeed, when TMRM-bound enzyme was added to TbGTP, the emission band at 488 nm was quenched when compared with the native unmodified enzyme. In this instance the donor of the fluorescence emission was not TbGTP alone but TbGTP complexed with adenylsuccinate synthetase. Resonance energy-transfer experiments show the distance between Cys$^{291}$ and the IMP binding site to be 17 Å and the distance from Cys$^{291}$ to the GTP binding site to be 9 Å. Energy-transfer was also seen between the tryptophanyl moiety of the enzyme and the chromophore label on Cys$^{291}$.

Previous studies involving quenching of the intrinsic fluorescence by substrates indicated the possibility of one or more tryptophan residues being located in the active site of the enzyme (17). Comparison of the amino acid sequence of E. coli adenylsuccinate synthetase with the other known sequences of the enzyme from mouse muscle and from D. discoideum (Table I) revealed 3 of the 4 Trp residues are in highly conserved regions (18, 19). Since attempts to study the role of the Trp residues by chemical modification using a Trp specific reagent, N-bromosuccinimide, revealed that all the Trp residues in adenylsuccinate synthetase are "buried" and inaccessible to the reagent, site-directed mutagenesis offered a more
convenient method to gain insight into this matter. The results of the
mutagenesis of Trp$^{309}$ to Phe and Trp$^{373}$ to Phe separately, revealed a slight
decrease in the $k_{cat}$s of the mutants and no significant changes in the $K_m$s
of the mutant enzymes.
Table I: Comparison of sequences surrounding residues 309 and 378 in *E. coli* and other adenylosuccinate synthetases

<table>
<thead>
<tr>
<th>enzyme</th>
<th>residues around 309</th>
<th>residues around 378</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGAS</td>
<td>T G R R R T G W L D</td>
<td>V E P I Y E T M P G W S</td>
</tr>
<tr>
<td>DDAS</td>
<td>T G P R R I G W L D</td>
<td>C E V V Y E S F P G W K</td>
</tr>
<tr>
<td>MMAS</td>
<td>T G K R R C G W L D</td>
<td>V E V E Y E T L P G W K</td>
</tr>
</tbody>
</table>

*a* EGAS = *E. coli* adenylosuccinate synthetase, DDAS = *D. discoideum* adenylosuccinate synthetase, MMAS = mouse muscle adenylosuccinate synthetase.

*b, c* the underlined residues are conserved
EXPERIMENTAL PROCEDURES

Materials: IMP, GTP, GDP, AMP, FMP, L-aspartate, adenylosuccinate, guanidium hydrochloride, and 1,5-I-AEDANS were obtained from Sigma Chemical Co. TMRM was from Molecular Probes. Restriction enzymes were obtained from United States Biochemicals and New England Biolabs. T4 DNA ligase and T4 polynucleotide kinase were from New England Biolabs. Oligonucleotides were synthesized by the Iowa State University Nucleic Acid Facility using a Biosearch 8570EX automated DNA synthesizer. Terbium (III) chloride hexahydrate and quinine sulfate monohydrate were from Aldrich. Distilled, deionized water was used exclusively. All other chemicals were obtained from commercial sources and were of the highest quality available.

Preparation and modification of adenylosuccinate synthetase:
Adenylosuccinate synthetase was prepared from E. coli and the purity and activity assays were performed as described previously (17). The purified enzyme was dialyzed against 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA at 4°C before any modification experiments were done. Chemical modification experiments with 1,5-I-AEDANS and TMRM were performed as described previously (17). The enzyme was dialyzed extensively against 50 mM potassium phosphate buffer to remove excess modifying reagent. The modified enzymes were analyzed for cysteinyl residues using DTNB after exposure to 8 M urea to check the efficiency of the modifying procedure. The absence of a single cysteinyl residue in the mutant enzymes was confirmed by this chemical analysis. The AEDANS-modified enzyme retained 97% of its activity and the TMRM-modified enzyme retained 69% of its activity compared to the control, as determined from enzyme activity assays.
after chemical modification.

Chemical modification of adenylosuccinate synthetase using N-bromosuccinimide: To 1.25 mg enzyme in 1 ml of 0.05 M sodium acetate buffer, pH 4 or 5.5, was added small aliquots of an aqueous stock solution of 10 mM N-bromosuccinimide and the absorbance of the resulting solution was determined at 280 nm after each addition (20). The following equation can be used to calculate the number of Trp residues modified/mole (20):

\[
n = \frac{\Delta A_{OD} \times 1.31 \times MW \times V}{W \times 5500}
\]

where,
- \(\Delta A_{OD}\) = corrected decrease in absorbance at 280 nm
- MW = molecular mass of protein
- V = initial volume of titrated solution
- 5500 = \(\varepsilon\) at 280 nm for Trp
- 1.31 = empirical factor which corrects for the absorption at 280 nm of the oxidation product of tryptophan, oxindole

Unfortunately, there was no significant decrease in absorbance on addition of N-bromosuccinimide to the enzyme solution. In fact, after an extremely small decrease initially, the absorbance actually increased. Hence, this method could not be used to modify the Trp residues since the extent of modification could not be quantitatively estimated.

Fluorescence measurements: Three distance measurements were made:

1) From the IMP binding site to Cys\(^{291}\) in the presence and absence of GTP. In this instance, FMP, a fluorescent analog of AMP, was used. Both AMP and FMP are competitive inhibitors of IMP and presumably bind at the IMP site.
FMP was the energy donor, and 1,5-I-AEDANS, which was used to modify Cys^{291}, was the energy acceptor.

2) From the GTP binding site to Cys^{291} in the presence and absence of IMP. In this instance, TbGTP was the energy donor, and TMRM, covalently bound to Cys^{291}, was the energy acceptor.

3) From the tryptophanyl moiety of the enzyme to Cys^{291}. The intrinsic protein fluorescence of the enzyme was the energy donor and 1,5-I-AEDANS was the acceptor.

All fluorescence measurements were made on a SLM 8000C spectrofluorometer as described earlier (17). Quantum yields were determined relative to a value of 0.55 for quinine sulfate in 0.1 N H_{2}SO_{4} using an exciting wavelength of 350 nm. The comparative relationship of Parker and Rees (21) was used:

\[
\frac{Q_2}{Q_1} = \frac{(F_2 \cdot A_1)}{(F_1 \cdot A_2)}
\]  

\(Q_2\) is the quantum yield of the unknown, \(F_2\) the area of the corrected emission spectrum, and \(A_2\) the absorbance at the exciting wavelength. \(Q_1\), \(F_1\), and \(A_1\) are the corresponding values for the standard quinine sulfate.

The mutant enzymes were first diluted with 25 mM Hepes, pH 7.7, to a final concentration of 0.5 \(\mu M\) (Bradford) and the intrinsic fluorescence was measured. Spectra were recorded and corrected using a buffer blank. In order to record the fluorescence spectra of the denatured protein, the enzymes were diluted with 6.4 M guanidine hydrochloride in 0.1 M Tris-HCl, pH 7.5, to a final concentration of 0.5 \(\mu M\). Spectra were recorded after one hour, after correction with respect to a buffer blank containing 6.4 M guanidine hydrochloride (22).
Samples used for fluorescence measurements usually had an absorbance below 0.05 at the exciting wavelength to avoid inner-filter quenching. Except in the case of TbGTP, the concentration of the acceptor was at least four times greater than that of the donor. In the case of TbGTP, in which this ratio could not be maintained without increasing the absorbance of the solution to above 0.05, a correction was made for quenching due to energy transfer from the freely diffusing energy donors to acceptors.

**Site-directed mutagenesis:** A 1.6-kb HindIII fragment of pMS204 containing part of the adenylosuccinate gene was ligated into the HindIII site of pUC118. The ligation mixture was used to transform competent TGI cells. Colonies were isolated from LB plates containing 40 µg/ml ampicillin. Nine colonies were chosen and plasmid was isolated from each colony and digested with EcoRI and HindIII. A plasmid of the proper size and orientation was used for DNA sequencing and site-directed mutagenesis.

The following oligonucleotides were prepared for the Cys$^{344}$ to Ser and Cys$^{344}$ to Ala mutations: (a) mutagenic oligonucleotide, dTAAGCCACGG*AGAGTTTAA. This had a G → C base pair change which substituted a Cys at residue 344 in the wild-type adenylosuccinate synthetase with a Ser. (b) Mutagenic oligonucleotide, dTAAGCCACGG*C*GAGTTTAA. This oligonucleotide had a 2 base pair change which substituted Cys$^{344}$ with an Ala. (c) Sequencing oligonucleotide, dAGACCACCGGA. This oligonucleotide primes 80 nucleotides upstream from the first nucleotide in the mutagenic oligonucleotide.

The following oligonucleotides were used to direct the mutations involving Trp$^{309}$ and Trp$^{378}$ respectively:
Sanger sequencing was performed using the mutagenic oligonucleotides as primers (23). Oligonucleotide (a), (1), and (2) annealed to only the desired site as seen by the clear and unambiguous sequencing results. Site-directed mutagenesis was accomplished by the method of Nakamaye and Eckstein (24). The presence of the mutation was confirmed by DNA sequencing. Oligonucleotide (b) did not anneal well to the wild-type HindIII fragment inserted in pUC118 but it did anneal well to the fragment with the Ser mutation, hence the Ala mutant was prepared using the fragment with the Ser mutation as the template strand and oligonucleotide (b) as the primer. DNA sequencing confirmed the presence of the Ala mutant. The HindIII fragments encoding the Ser, Ala and the Phe mutations were each cloned back into pMS 204 and the resulting plasmids were designated pCS100, pAlal, pW309F and pW378F respectively. TG1 cells were transformed using these mutant constructs. Colonies were selected from LB plates containing chloramphenicol (26 μg/ml). Plasmid was prepared by the method of Birnboim and Doly (25) from a few of the colonies obtained after transformation, and a colony containing a plasmid with the correct orientation and size (as confirmed by digestion with EcoRI and HindIII) was used as a source of each of the mutant forms of the enzyme. The mutant forms of adenylosuccinate synthetase were purified using protocols identical to those reported for
the wild-type enzyme (2). The mutant enzymes were analysed for cysteinyl residues using DTNB after exposure to 8 M urea. The absence of a single cysteinyl residue in the mutant enzymes was confirmed by this chemical analysis. The absence of a single Trp residue in the mutant enzymes was revealed in the fluorescence spectra of the enzymes in 6.4 M guanidine hydrochloride (22).

**Electrophoresis:** Restriction enzyme digestions on the plasmid DNA were analyzed by electrophoresis on agarose gels in Tris-borate-EDTA buffer. The gels were stained and visualized by ethidium bromide (26).
Energy transfer: The efficiency of energy transfer (T), when the enzyme is saturated with the energy acceptor, is given by

\[ T = 1 - \frac{Q_{da}}{Q_d}, \]  

where \( Q_{da} \) and \( Q_d \) are the quantum yields of donor in the presence and absence of acceptor, respectively. The energy-transfer efficiency (T) is related to the distance between the chromophores according to the following relationship (Förster, 1948) (27-29):

\[ R = R_o \left( \frac{1}{T - 1} \right)^{1/6}, \]  

where \( R_o \) is the Förster critical distance for a given donor and acceptor pair.

\( R_o \) is calculated according to the following equation:

\[ (R_o)^6 = (8.79 \times 10^{-25})k^2n^{-4}Q_dJ_{da}, \]  

where \( J_{da} \) is the spectral overlap integral, \( n \) is the refractive index of the medium (1.33), \( Q_d \) is the quantum yield of the donor in the absence of the acceptor, and \( k^2 \) is the orientational factor which is defined as (29):

\[ k^2 = (\cos \theta_t - 3\cos \theta_d \cos \theta_a)^2 \]  

where \( \theta \) is the angle between emission dipole of the donor and absorption dipole of the acceptor, and \( \theta_d, \theta_a \) are the angles between dipoles and vector connecting donor and acceptor. \( k^2 \) can have values from 0 and 4.0. \( k^2 \) equals 4 when the dipoles are parallel and oriented in the same direction and \( k^2 \) equals 0 only in the case of perpendicular orientation. When the donor and acceptor rotate rapidly relative to the fluorescence lifetime of the donor, \( k^2 \) is taken to be 2/3 (i.e. assuming that the donor and the acceptor undergo complete dynamic isotropic orientational averaging) (30).
In this report, in one instance, the donor was FMP, and the acceptor was 1,5-I-AEDANS; in the second case, the donor was the TbGTP-enzyme complex, and the acceptor was TMRM; in the last study, the donor was the tryptophanyl moiety of the enzyme, and the acceptor was 1,5-I-AEDANS. The orientation factor $k^2$, usually taken as $2/3$ is the uncertain factor in the $R_0$ calculation. In the second instance, in which the donor is a metal ion, the degeneracy of the excited state makes the $k^2$ value of $2/3$ a good approximation making the maximum error limit in the distance measurement within 10% (6, 7, 31). Because of the sixth-root dependence of $R_0$, the discrepancy in the values of $k^2$ or $n$ are not reflected to a great extent in the determination of $R_0$. 
RESULTS

Modification of Cys$^{344}$ by NEM caused a complete loss of enzyme activity (3). This loss of activity may be due to direct involvement of Cys$^{344}$ at the active site of the adenylosuccinate synthetase, to involvement of Cys$^{344}$ at an essential site other than the active site, or to alterations of the native conformation induced by the chemical modification. In order to make a choice from among these possibilities, site-directed mutagenesis experiments in which Cys$^{344}$ was replaced by either a seryl or an alanyl residue were undertaken. The mutant enzymes were isolated using protocols identical to those described for the wild-type enzyme. It was observed that there are no significant alterations in the kinetics (specific activities and Michaelis constants, data not shown) of adenylosuccinate synthetase among the wild-type and mutant enzyme forms. These findings strongly suggest that Cys$^{344}$ is not essential for the catalytic functioning of adenylosuccinate synthetase. A comparison of the alanine mutant and the wild-type enzyme indicates that Cys$^{344}$ probably does not play a structural role, through hydrogen bonding, in adenylosuccinate synthetase. These site-directed mutagenesis studies lead to the conclusion that reaction of Cys$^{344}$ with NEM causes enzyme inactivation because the modified enzyme is unable to assume its native conformation and not because Cys$^{344}$ is either involved in catalysis directly or required to maintain the structure of the native enzyme.

We recently reported that adenylosuccinate synthetase-nitroxide modified Cys$^{291}$ was greater than 9.8 Å from the GTP binding site (3). Experiments involving covalent modification by pyridoxal-5'-phosphate of
lysyl residues and their protection by GTP indicated that Lys$^{140}$ was an important locus for GTP binding (32). This hypothesis was supported by studies in which Lys$^{140}$ was mutated to Ile. Although these investigations seem to pinpoint the location of the adenylosuccinate synthetase GTP binding site, they clearly do not represent the definitive study on the location of the active site of the enzyme. Adenylosuccinate synthetase was recently crystallized and X-ray diffraction analysis studies have been carried out in a collaborative effort with Dr. R. B. Honzatko (33).

Experiments by Kim (34) indicate that the distance from mercury iodide modified Cys$^{291}$ to the tentative binding site for aspartate is approximately 15 Å. Unfortunately, it has been observed that GTP, GDP and IMP cause shattering of the adenylosuccinate crystals (35), and definitive information on the binding site for GTP may not be available in the near term. It was for this reason that we attempted to measure the distance from Cys$^{291}$ to the active site of adenylosuccinate synthetase using an alternative spectroscopic method. The technique of fluorescence spectroscopy which under favorable conditions permits one to determine distances between two fluorophores over relatively long distances was chosen. Two conditions have to be fulfilled in order to perform energy transfer measurements of labeled proteins, (1) the fluorescence donor emission should overlap the fluorescence absorbance of the acceptor and (2) the protein should bind the chromophore or be covalently attached to it at a unique location so that the distance of a distinct amino acid to the fluorescence acceptor is obtained (29). Both these conditions were fulfilled in the case of 1,5-I-AEDANS and TMRM modification of
adenylosuccinate synthetase. Under these conditions, as seen from Table II, the distance from the IMP binding site to Cys\textsuperscript{291} is 17 Å, and the distance from the GTP binding site to Cys\textsuperscript{291} is 9 Å.
TABLE II: Energy Transfer to the Chromophore at Cys^{291}

<table>
<thead>
<tr>
<th>Distance to</th>
<th>$J$ (M$^{-1}$ cm$^3$)</th>
<th>$T$</th>
<th>$r$ (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>measured</td>
<td>spectral overlap integral</td>
<td>energy-transfer efficiency</td>
<td>distance between donor and acceptor</td>
</tr>
<tr>
<td>Cys^{291} to GTP site</td>
<td>$1.64 \times 10^{-14}$</td>
<td>0.56</td>
<td>9</td>
</tr>
<tr>
<td>Cys^{291} to IMP site</td>
<td>$7.57 \times 10^{-16}$</td>
<td>0.39</td>
<td>17</td>
</tr>
<tr>
<td>Cys^{291} to Trp moiety</td>
<td>$6.39 \times 10^{-16}$</td>
<td>0.34</td>
<td>18</td>
</tr>
</tbody>
</table>
Like other proteins, adenylosuccinate synthetase has an intrinsic fluorescence associated with its aromatic amino acids and, at pH 7.7 the excitation and emission maxima are 280 and 333 nm, respectively (17). The emission band coincides with the absorption maximum of the bound 1,5-I-AEDANS at 337 nm. This suggested that resonance energy transfer from the tryptophan moiety to 1,5-I-AEDANS is possible. In fact, when adenylosuccinate synthetase modified at Cys\(^{291}\) with 1,5-I-AEDANS is excited at 280 nm, it fluoresces not only at 333 nm, but also at 480 nm indicating the occurrence of energy transfer from the tryptophanyl moiety to 1,5-I-AEDANS. Assuming that energy transfer is the only reason for the decrease in quantum yield between the native enzyme and the modified enzyme, a transfer efficiency of 0.34 was calculated leading to a distance of 18 Å from the tryptophanyl moiety to Cys\(^{291}\).

Fluorescence emission spectra of proteins with tryptophan residues present is almost entirely due to the tryptophan residues (36). The observed fluorescence can be almost exclusively attributed to tryptophan if the protein were excited at 295 nm, because, at 295 nm only tryptophan residues are excited. Previous experiments showed that the enzyme when excited at 295 nm, fluoresced at 333 nm and this fluorescence was quenched by some active site binding ligands (17). Thus, it was possible that one or more Trp residues were present at the active site and might be important for substrate binding or catalysis. Another explanation is that ligand binding induces conformational changes that affect Trp residues that are distal to the active site. Tryptophan residues in proteins can be modified and quantitated by using N-bromosuccinimide which is quite specific for Trp.
residues and the importance of the Trp residues could be determined similar to the way the Cys residues had been chemically modified (20, 3). However, this protocol didn't work in the case of adenylosuccinate synthetase (see experimental procedures for details). Therefore site-directed mutagenesis of Trp$^{309}$ and Trp$^{378}$ to Phe, one at a time, was resorted to in order to determine the status of the Trp residues. The results of the kinetic experiments involving the wild-type and mutant enzymes are given in Table III. As can be seen from the Table the $k_{cat}$s of the mutants are decreased by 28%. Apart from this minor change there are no significant changes in the Michaelis constants leading us to believe that neither of these residues are involved in substrate binding or catalysis. They might be important in maintaining the structure of the enzyme and this may lead to the slight decrease in the $k_{cat}$s of the mutants that are observed (Table III). The emission spectra of the wild-type and the mutants under non-denaturing conditions are shown in Figure I. The position of the maximum emission wavelength and also the intensity of the fluorescence varies slightly between the mutants and this variation reflects the subtle differences in the microenvironment of each Trp (Table IV). The spectrum of the mutant with Trp$^{378}$ shows a slight red shift and a lower intensity compared with the mutant with Trp$^{309}$ indicating that Trp$^{378}$ is more accessible to solvent than Trp$^{309}$ and that Trp$^{309}$ is in a slightly more hydrophobic environment than Trp$^{378}$. The emission spectra in Figure II under denaturing conditions show a stepwise loss of intensity due to the replacement of one Trp residue by Phe. The spectra shown are those taken an hour after dilution of the protein with guanidium hydrochloride but similar
spectra were obtained immediately after, and 24 hours after denaturation which shows that the enzyme and its mutants are completely denatured very rapidly unlike other proteins that require hours to days for total denaturation under the above conditions (20).
Figure 1. Fluorescence emission spectra of adenylosuccinate synthetase and its tryptophan mutants. The protein was contained in 25 ml Hepes buffer, pH 7.7. Fluorescence excitation was at 280 nm. (A) wild type enzyme; (B) W309F; (C) W378F.
Figure 2. Fluorescence emission spectra of adenylosuccinate synthetase and its tryptophan mutants under denaturing conditions. The protein was contained in 6.4 M guanidine hydrochloride in 0.1 M Tris-HCl, pH 7.5. (A) wild type enzyme; (B) W309F; (C) W378F.
Table III: Kinetic properties of the wild type and Trp mutants of adenylosuccinate synthetase.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Wild Type</th>
<th>W309F</th>
<th>W378F</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_{cat}(s^{-1})$</td>
<td>1.91 ± 0.02</td>
<td>1.40 ± 0.01</td>
<td>1.39 ± 0.07</td>
</tr>
<tr>
<td>$K_m^{GTP}$ ($\mu$M)</td>
<td>10.6 ± 0.8</td>
<td>10.7 ± 1.1</td>
<td>10.4 ± 1.9</td>
</tr>
<tr>
<td>$K_m^{IMP}$ ($\mu$M)</td>
<td>21.1 ± 2.1</td>
<td>20.7 ± 3.8</td>
<td>18.2 ± 2.0</td>
</tr>
<tr>
<td>$K_m^{Asp}$ ($\mu$M)</td>
<td>141 ± 18</td>
<td>346 ± 87</td>
<td>126 ± 18</td>
</tr>
<tr>
<td>$k_{cat}/K_m^{GTP}(s^{-1}M^{-1}) \times 10^6$</td>
<td>18.0 ± 1.3</td>
<td>13.4 ± 1.3</td>
<td>13.3 ± 2.4</td>
</tr>
<tr>
<td>$k_{cat}/K_m^{IMP}(s^{-1}M^{-1}) \times 10^6$</td>
<td>9.05 ± 3.20</td>
<td>6.76 ± 1.23</td>
<td>7.63 ± 0.92</td>
</tr>
<tr>
<td>$k_{cat}/K_m^{Asp}(s^{-1}M^{-1}) \times 10^6$</td>
<td>1.35 ± 0.17</td>
<td>0.40 ± 0.10</td>
<td>1.10 ± 0.16</td>
</tr>
<tr>
<td>protein</td>
<td>native (nm)</td>
<td>denatured (nm)</td>
<td></td>
</tr>
<tr>
<td>-------------</td>
<td>-------------</td>
<td>----------------</td>
<td></td>
</tr>
<tr>
<td>wild type</td>
<td>333</td>
<td>349</td>
<td></td>
</tr>
<tr>
<td>W309F</td>
<td>337</td>
<td>350</td>
<td></td>
</tr>
<tr>
<td>W378</td>
<td>333</td>
<td>350</td>
<td></td>
</tr>
</tbody>
</table>
DISCUSSION

One of the primary reasons for undertaking the site-directed mutagenesis experiments involving Cys$^{344}$ were the results from chemical modification of Cys$^{344}$ using NEM which selectively and specifically inactivated adenylosuccinate synthetase indicating that Cys$^{344}$ might be an important residue for substrate binding and/or for catalysis. However, the results from site-directed mutagenesis do not mimic these results. One reason may be that chemical modification might be causing a more significant change in the altered Cys residue than does the substitution of Ser or Ala, i.e., the structure of the enzyme is subjected to a significantly more profound effect by the addition of bulky thiol reagents than does the substitution of Cys by Ser or Ala. Another reason might be that the mutations require the protein to fold properly in the presence of the mutant amino acid, whereas the partially folded protein was chemically modified. Yet another possibility is that Cys$^{344}$ lies at or near important points of contact between the two subunits of the dimer. Chemical modification of Cys$^{344}$ might cause an increase in the side chain of this amino acid and thus cause a larger change in the structure of the protein than does Ser or Ala which have a relatively minor size difference with regard to Cys at position 344 and hence do not affect the structure of the subunit.

An interesting property of adenylosuccinate synthetase modified with 1,5-I-AEDANS is the occurrence of resonance energy transfer in the enzyme. The enzyme gives rise to a fluorescence at 333 nm when excited at 280 nm because of the aromatice amino acid residues in the protein, and also to a
1,5-I-AEDANS fluorescence at 480 nm. The efficiency of energy transfer was calculated to be 0.34 leading to a $R_o$ value of 16 Å (Table II). Because there are four Trp residues in the enzyme, it is not possible to deduce from these data any information about distances or orientations of the chromophores involved. Nevertheless, because the efficiency of transfer depends on the inverse of the sixth power of the distance between the chromophores, the results suggest that the nearest Trp residue is at least 18 Å away from Cys$^{291}$. This finding represents an "average" distance between the sulfhydryl and the tryptophan residues and provides a qualitative estimate for the distance over which energy transfer might occur.

By chemical modification studies using N-bromosuccimide, tryptophan has been shown to be involved in the function of enzymes such as hen egg lysozyme (37), $\alpha$-chymotrypsin (38), trypsin (39), $\alpha$-amylase (40), pepsin (41), and in the binding sites of proteins like avidin (42). However, some proteins, including adenylosuccinate synthetase, possess tryptophan residues which resist oxidation by NBS, reacting very slowly or not at all, under the usual conditions (43, 44). This behaviour is consistent with tryptophan residues "buried" in hydrophobic regions of the protein and can be exposed to the reagent only in concentrated urea, under conditions which affect the tertiary structure of proteins as in avidin, tobacco mosaic virus protein, some hemoglobins and glutamic dehydrogenase (43-45). Previously we demonstrated that the addition of substrates like IMP, GTP, and products like adenylosuccinate cause quenching of the intrinsic fluorescence of adenylosuccinate synthetase. Quenching of fluorescence in
the presence of substrates suggests that tryptophan and the ligand might possibly be interacting directly. On the other hand, these results may be interpreted to mean that there is a change in the enzyme conformation caused by the binding of substrate to the active site and this alters the environment near the fluorescing tryptophan residue thus causing quenching. The tryptophan(s) whose fluorescence is being quenched need not be in proximity to the active site. There are four Trp residues in the sequence of adenylosuccinate synthetase. Three of the four Trp residues are conserved in the synthetases from both *D. discoideum* and mouse muscle (18, 19) (Table I). Thus, it might be anticipated that if each of these three conserved residues were mutated to another amino acid, at least one form of adenylosuccinate synthetase would result which would differ significantly in activity from the wild type. The results of the mutation of two of the Trp residues reported are contrary to this expectation because their activity does not differ significantly from the wild type (Table IV). This result and the fact that the fluorescence emission spectra of the mutants are slightly different from the wild-type (Fig. I) leads to the conclusion that Phe is much like Trp that, except for a small change, this replacement does not cause any destabilization of the protein. The results of the mutation of the third conserved Trp residue might prove different.

Tryptophan fluorescence in proteins is heterogenous because each residue has a different maxima and quantum yield depending on the microscopic environments of the residues and its degree of exposure to solvents (36, 22). "Buried" tryptophans in proteins fluoresce maximally at around 330 nm, while tryptophans in proteins that are exposed fluoresce
maximally at lower frequencies (46, 47). The fluorescence band shapes are usually broad and overlap, in most cases very little information can be gained about individual tryptophans. Therefore, site-directed mutagenesis of Trp residues is proving to be helpful in the interpretation of the intrinsic protein fluorescence. Although they do not differ much in activity, the mutant enzymes seem to have slightly different fluorescent properties and analysis of the fluorescent spectra reveal that the microenvironments of these amino acid residues are slightly different (Fig I). From the position of the emission peak of W378F it would appear that Trp^{309} is located towards the interior of the molecule whereas Trp^{378} might be more exposed to the solvent than Trp^{309} (Table III). Knowledge of the three-dimensional structure of adenylosuccinate synthetase would confirm this.

Modification of Cys^{291} with AEDANS and TMRM permitted us to measure the distance from these fluorophores to the active site binding ligands, TbGTP and FMP. It was found that the distance from Cys^{291} modified with AEDANS to the IMP binding site is 17 Å, whereas TMRM bound to Cys^{291} is 9 Å from the GTP site (Table II). All distance measurements were made assuming a single donor-acceptor pair. Because the enzyme is a homodimer, there are actually two acceptors and donors present. It is possible that the distances measured are intersubunit rather than intrasubunit in which case the distances calculated would be somewhat longer than calculated if the interaction between energy donors and acceptors is important. Therefore, the distances calculated can be regarded as lower bounds.

One of the most striking results emerging from the present study is
the difference in the distance from Cys$^{291}$ to the IMP binding site (17 Å) and from Cys$^{291}$ to the GTP binding site (9 Å). Previous results from this laboratory using a spin-labeled analog of N-ethylmaleimide and using NMR techniques indicated the distance between Cys$^{291}$ and the GTP binding site was $> 9.8$ Å (3). This distance, however, is from the nitroxide on the spin label at Cys$^{291}$ to the H-8 proton of GTP. The distance of 9 Å from Cys$^{291}$ to the Tb bound to the phosphate of GTP does not contradict previous results because in the syn conformation (M. Roy and Fromm, unpublished observations), TbGTP should be greater than 10 Å in length. In the mechanism proposed for adenylosuccinate synthetase in which 6-phosphoryl-IMP is an obligatory intermediate (48), it might be expected that the GTP and IMP sites within the active site are juxtaposed. The distance differences cited here may be rationalized in a number of ways. First, it is unlikely that the fluorophores covalently bound to Cys$^{291}$ occupy precisely the same positions on the enzyme, because of their different sizes. Second, because GTP is in the syn conformation this factor may account for the differences in distances for the two ligands. The conformation of FMP and 1,5-I-AEDANS are unknown. Furthermore, it should be noted that in our measurements we have not corrected our distance estimates with regard to the probe size or the point of attachment of the probe to the enzyme. Future investigations will be required to resolve these distance differences.
REFERENCES


22. Pace, C. N. Methods Enzymol. 131, 266-279.


46. Hewlett, E. L., Gordon, V. M., McCaffery, J. D., Sutherland, W. M., and


GENERAL SUMMARY

TbGTP and TbGDP proved to be sensitive probes of ligand binding to adenylosuccinate synthetase and changes in their fluorescence on complexing with the enzyme and various ligands made it possible to detect subtle conformational changes occurring at the active site. This dissertation has provided some more evidence to show that IMP and aspartate have binding sites that are spatially separated and that GTP and GDP induce different conformational states when bound to the enzyme so that 6-phosphoryl-IMP is very likely an intermediate in the adenylosuccinate reaction mechanism.

Information on the spatial relationship between Cys\textsuperscript{291} and the IMP and GTP binding sites was obtained using fluorescence resonance energy transfer. Site-directed mutagenesis studies showed that Cys\textsuperscript{344}, Trp\textsuperscript{309} and Trp\textsuperscript{378} are not required for either substrate binding or catalysis although the latter two amino acid residues may be involved in maintaining the proper structure of the enzyme.

Knowledge of the three-dimensional structure of the enzyme would be very helpful in performing further experiments to determine which amino acids are involved in substrate binding and/or catalysis.
LITERATURE CITED


I would like to express my sincere gratitude to my major professor, Dr. H. J. Fromm, for his support and guidance through the course of my stay at ISU. I am grateful to Dr. Alan M. Myers for his help and guidance with the mutagenesis experiments. Thanks also to all the members of his lab for their friendliness, help and patience.

I would like to thank Dr. D. J. Graves, Dr. B. J. White, Dr. R. B. Honzatko and Dr. E. S. Wurtele for serving on my committee.

Special thanks to all the members of my lab for their cooperation and valuable suggestions.

Finally, I would like to express my gratitude to my parents, Hannah and Clement, my sister, Ramola, and my brother, Ashwin, for their love, support and prayers without which this would not have been possible.