The Use of a Specific Fluorescence Probe to Study the Interaction of Adrenodoxin with Adrenodoxin Reductase and Cytochrome P-450_{sec}*

(Received for publication, February 24, 1987)

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Adrenodoxin is a small, highly acidic iron-sulfur protein that transfers electrons from NADPH-adrenodoxin reductase to cytochromes P-450_{sec} and P-450_{1a} in adrenal cortex mitochondria. Cytochrome P-450_{sec} carries out the side chain cleavage of cholesterol to form pregnenolone, while cytochrome P-450_{1a} participates in steroid 11β hydroxylation. Lambeth and co-workers (1979, 1983) have proposed that adrenodoxin functions as a mobile shuttle in transporting electrons from adrenodoxin reductase to cytochrome P-450_{sec}. This is supported by the finding that oxidized adrenodoxin forms a tight 1:1 complex with adrenodoxin reductase at low ionic strength. Reduction of adrenodoxin then lowers the binding constant for this complex significantly, favoring dissociation. In a similar fashion, reduced adrenodoxin forms a tight 1:1 complex with cytochrome P-450_{sec}. Following electron transfer, the oxidized adrenodoxin binds less tightly to the P-450 and is released to bind once again to the reductase (Hanukoglu and Jefcoate, 1980; Light and Orme-Johnson, 1981). The binding of adrenodoxin to both adrenodoxin reductase and cytochrome P-450_{sec} is strongly inhibited by ions, suggesting the importance of electrostatic interactions (Lambeth et al., 1979; Lambeth and Kriengsiri, 1985). We have recently used the water-soluble carbodiimide EDC¹ to modify carboxyl groups at residues 72, 73, 74, 76, 79, and 86 on adrenodoxin (Geren et al., 1984). This modification inhibited the interaction with both adrenodoxin reductase and cytochrome P-450_{sec} to the same extent. Since these groups are located close to one another on the surface of adrenodoxin, the binding domains for the reductase and the P-450 appear to be nearly the same, providing additional evidence that adrenodoxin functions as a mobile shuttle. We also found that the water soluble carbodiimide could cross-link adrenodoxin to adrenodoxin reductase or cytochrome P-450_{sec} to form covalently linked binary complexes (Lambeth et al., 1984). However, when a mixture of all three proteins was treated with the carbodiimide, no ternary complexes were formed.

In contrast to the above, Kido and Kimura (1979) have reported that a stable ternary complex of the three proteins was detected by difference spectroscopy and gel chromatography. However, it was not established whether this complex was catalytically active. Usanov et al. (1985) have recently reported that a covalently cross-linked adrenodoxin-adrenodoxin reductase complex retained some electron transfer activity with cytochrome P-450_{sec}, suggesting that different domains on adrenodoxin are involved in reacting with the reductase and the P-450. A similar conclusion was reached by Chashchin et al. (1985), who reported that a cross-linked complex of adrenodoxin and cytochrome P-450_{sec} was able to accept electrons from adrenodoxin reductase. However, in both of these studies a 13 Å long reagent was used to cross-link a lysine amino group on adrenodoxin to a lysine on the partner. This length could allow the cross-linked adrenodoxin to sufficient rotational flexibility to transfer electrons from the reductase to cytochrome P-450_{sec}, particularly if the cross-link was not located at the reaction domain on adrenodoxin.

In the present paper we report that labeling the single free cysteine at residue 95 of adrenodoxin with the fluorescent reagent N-iodoacetylaminooxy-1-aminonaphthalene-5-sulfonate (1,5-I-AEDANS) did not alter the binding site for these molecules. Addition of adrenodoxin reductase, cytochrome P-450_{sec}, or cytochrome c to AEDANS-adrenodoxin resulted in the formation of 1:1 binary complexes. Förster energy transfer calculations indicated that the AEDANS label on adrenodoxin was 42 Å from the heme group in cytochrome c, 36 Å from the FAD group in adrenodoxin reductase, and 58 Å from the heme group in cytochrome P-450_{sec} in the respective binary complexes. These studies suggest that the FAD group in adrenodoxin reductase is located close to the binding domain for adrenodoxin but that the heme group in cytochrome P-450_{sec} is deeply buried at least 26 Å from the binding domain for adrenodoxin. Modification of all the lysines on adrenodoxin with maleic anhydride had no effect on the interaction with either adrenodoxin reductase or cytochrome P-450_{sec}, suggesting that the lysines are not located at the binding site for either protein. Modification of all the arginine residues with p-hydroxyphenylglyoxal also had no effect on the interaction with adrenodoxin reductase or cytochrome P-450_{sec}. These studies are consistent with the proposal that the binding sites on adrenodoxin for adrenodoxin reductase and cytochrome P-450_{sec} overlap, and that adrenodoxin functions as a mobile electron carrier.

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* This work was supported by National Science Foundation Grant PCM-8314707 (to F. M. and L. G.), and National Institutes of Health Grant RR07101 (to F. M.) and AM27373 (to J. D. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1794 solely to indicate this fact.

¹ The abbreviations used are: EDC, 1-ethyl-3-(dimethylaminopropyl)carbodiimide; 1,5-I-AEDANS, N-iodoacetylaminooxy-1-aminonaphthalene-5-sulfonate; MOPS, 4-morpholinepropanesulfonic acid; DTT, dithiothreitol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high pressure liquid chromatography.
reagent, 1,5-I-AEDANS had no effect on the interaction with adrenodoxin reductase or cytochrome P-450. Addition of adrenodoxin reductase, cytochrome P-450, or cytochrome c to AEDANS-adrenodoxin was found to quench the fluorescence in a manner consistent with the formation of 1:1 binary complexes. Förster energy transfer calculations were then carried out to determine the distance from the AEDANS label on adrenodoxin to the heme groups in cytochrome P-450, and cytochrome c, and to the flavin in adrenodoxin reductase. We also report that modification of all the lysines on adrenodoxin with maleic anhydride had no effect on the interaction with either adrenodoxin reductase or cytochrome P-450, suggesting that lysines are not located at the binding site for either protein. Our studies are consistent with the proposal that the binding sites on adrenodoxin for reductase and P-450 overlap, and that adrenodoxin functions as a mobile electron carrier.

EXPERIMENTAL PROCEDURES

Materials—Horse heart cytochrome c (Type VI), MOPS, Hepes, 1,5-I-AEDANS, cholesterol, DTT, pyrrolidin-2-one, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, NADP, DTT, cholesterol, NADH, and 15-CI-5-fluorescein were obtained from Sigma. Tris was obtained from Schwartz/Mann, and adrenodoxin was obtained from Scioen Biocemica and Research Products or was prepared by the procedure of Suhara (1972) as modified in our laboratory (Geren and Miller, 1981). Dioxane was purchased from Fischer and K-385 (p-hydroxyphenylglyoxal) from Pierce Chemical Co. Cytochrome P-450 was prepared by the method of Lambeth et al. (1979), while adrenodoxin reductase was prepared as described by Lambeth and Kamin (1979).

1,5-I-AEDANS Modification of Adrenodoxin—Native adrenodoxin (200 μM) was treated with 5-6.5 μM 1,5-I-AEDANS in 50 mM Tris, pH 8.0, for 2-3 h at 25 °C in the dark. The excess reagent was removed by dialysis in 50 mM Tris, pH 8.0. The percent modification was determined spectroscopically using a molar extinction coefficient of 25,000 M⁻¹ cm⁻¹ at 250 nm for 1,5-I-AEDANS and 14,000 M⁻¹ cm⁻¹ at 280 nm for adrenodoxin. In order to identify labeled residues, AEDANS-adrenodoxin was carboxymethylated by incubation for 30 min in 4 M urea, 20 mM DTT at 25 °C followed by treatment with 40 mM iodoacetamide for 1 h. Following dialysis the protein (1 mg/ml) was digested with 6% trypsin for 6 h in 100 mM bicine, pH 8.0, at 37 °C. The tryptic digest was separated on a Brownie R-500 column using a linear gradient from 50 mM sodium carbonate, pH 9.0, to 0.1 M sodium bicarbonate, pH 7.0, to 100% methanol. The gradients were monitored on a Spectra-Physics SP-8700 solvent delivery system, and the eluent was monitored at 210 and 230 nm using a BDH Tracor 970A and Spectroflow 757 variable wavelength detectors in series. The amino acid composition of each purified peptide was determined by hydrolyzing the sample in 6 M HCl containing 0.1% 2-mercaptoethanol for 22 h at 110 °C in an evacuated, sealed tube. The hydrolysates were injected into a microbore amino acid analyzer equipped with ninhydrin detection.

Modification of Lysine, Arginine, and Histidine Residues—Lysine residues were modified by treating 0.7 mg/ml adrenodoxin, or 0.25 M bicine, pH 8.5, with 8 μl of 1 M maleic anhydride in 1,4-dioxane, added in 6 additions over 1 h at 25 °C. The extent of modification was determined by TNBS analysis using the procedure of Habeeb (1966). Modification of arginine residues was carried out by adding 10-20 mg/ml p-hydroxyphenylglyoxal to 30 μM adrenodoxin in 0.2 M sodium carbonate, pH 9.0, 1 M KCl. The samples were passed through Bio-Gel P-4 columns to remove excess reagent, and spectra were recorded on a Cary 210 spectrophotometer. The number of modified arginine residues was calculated at 340 nm using a molar extinction coefficient of 18,300 M⁻¹ cm⁻¹ (Yamasaki et al., 1983). Histidine residues were modified by treating 30-40 μM adrenodoxin in 30 mM sodium phosphate, pH 7.0, 0.6 M NaCl with 6-9 mM diethylpyrocarbonate. The extent of modification was determined using a molar extinction coefficient of 3,200 M⁻¹ cm⁻¹ at 240 nm (Ovad et al., 1967). Adrenodoxin Reductase Assay—The reaction between adrenodoxin and adrenodoxin reductase was monitored by following the reduction of cytochrome c at 550 nm in the presence of a NADPH generation system as previously described (Light and Walsh, 1980 and Lambeth et al., 1979). The assay was carried out in 50 mM sodium phosphate, pH 7.0, using 0.3-3 μM adrenodoxin, 66 mM NADH, 27 μM cytochrome c, 12 μM NADP, 300 μM glucose-6-phosphate, and 0.1 unit/ml glucose-6-phosphate dehydrogenase. Cytochrome P-450 Binding Measurements—The binding of adrenodoxin to cytochrome P-450 was monitored by following the change in the Soret maximum at 418 nm (low spin) and 392 nm (high spin). The concentration of adrenodoxin was adjusted to give a 0.18-0.4 μM P-450 in 20 mM Hepes, pH 7.2, plus 0.1 mM DTT, 0.1 mM EDTA, 0.1% Tween 80, 50-75 mM sodium chloride, and 26 μM cholesterol. An equal concentration of adrenodoxin was also added to a reference cuvette containing only buffer to eliminate absorbance changes due to titrant. Spectra were recorded on a Cary 210 spectrophotometer, and the change in absorbance due to conversion from low to high spin was plotted as a function of adrenodoxin concentration. Dissociation constants were calculated using a nonlinear regression program adapted from Duggleby (1981).

Fluorescence Studies—Fluorescence spectra of AEDANS-adrenodoxin were recorded with a Perkin Elmer 650-40 fluorescence spectrophotometer using an excitation wavelength of 340 nm. Cytochrome c quenching curves were obtained by measuring the fluorescence of 0.05-0.2 μM AEDANS-adrenodoxin in 5 mM MOPS, pH 7.5, before and immediately after the addition of 0-4 μM cytochrome c. Disintegration of the iron-sulfur center of adrenodoxin due to cytochrome c reduction was found to be negligible during the time of the experiment (Manabe, 1974). Cytochrome c concentrations were calculated using a molar extinction coefficient of 10,600 M⁻¹ cm⁻¹ at 410 nm (Margoliash, 1959). The cuvettes were silanized with dichlorodi-methyl silane to minimize adsorption to cell walls. Cytochrome P-450 adrenodoxin fluorescence quenching studies were carried out in 5 mM MOPS, pH 7.5, using 0.5-1 μM AEDANS-adrenodoxin and 0-2 μM cytochrome P-450 reductase. The concentration of adrenodoxin reductase was determined using an extinction coefficient of 10,900 M⁻¹ cm⁻¹ at 450 nm. The quenching of AEDANS-adrenodoxin by reduced adrenodoxin reductase was studied in an anaerobic cuvette containing 5 mM MOPS, pH 7.5, 0.2 μM NADPH, 1 mM glucose-6-phosphate, 0.003 units/ml glucose-6-phosphate dehydrogenase, 0.66 μM AEDANS-adrenodoxin, and 0.02-3 μM cytochrome P-450. The fluorescence of AEDANS-adrenodoxin was corrected for the very small increase in fluorescence associated with the addition of adrenodoxin reductase in the solution. The cytochrome P-450 studies were done in 20 mM Hepes, pH 7.2, 0.75 mM sodium chloride, 0.1 mM EDTA, 0.1 mM DTT, 0.1% Tween 80, and 26-52 μM cholesterol. Reduced cytochrome P-450 was obtained by reduction with sodium dithionite or with 0.02 μM adrenodoxin reductase, 1 μM adrenodoxin, and the NADPH-generating system described above. The concentration of cytochrome P-450 was determined from the reduced CO minus reduced difference spectrum, using a difference extinction coefficient of 92,000 M⁻¹ cm⁻¹ for A₄₅₀-A₄₃₀ (Umura, 1964). The slight decrease in fluorescence caused by inner and outer filter effects was corrected as described by Polgar and Wolfenden (1966). Intensities were measured for reduced cytochrome c with a Cary 60 spectrophotometer, and the polarization measurements were made with an Ansinco Bowman or Perkin-Elmer 650-40 fluorescence spectrophotometer and were calculated using the equation: \( P = (I_p - I_o)/(I_p + I_o) \) where \( I_p \) and \( I_o \) refer to the fluorescence intensities in which the emission is polarized in a parallel or perpendicular fashion with respect to the polarization of the exciting light. Quantum yield was determined using quinine bisulfate in 0.1 N H₂SO₄ at a concentration such that its absorbance at 340 nm is equivalent to that of a 2.5 μM solution of AEDANS-adrenodoxin.

RESULTS

Specific Modification of Adrenodoxin with 1,5-I-AEDANS—Treatment of adrenodoxin with 5 mM 1,5-I-AEDANS was found to result in covalent labeling of adrenodoxin with 0.6-1.0 eq of 1,5-I-AEDANS, depending on the reaction time. HPLC analysis of a tryptic digest of AEDANS-adrenodoxin showed that a single peptide containing residues 90-98 was labeled with AEDANS (Fig. 1). This peptide contains two cysteine residues, Cys-92 and Cys-95 (Table I). Since Cys-95 is conserved in the amino acid sequence of putidaredoxin, where it is known to bind the Fe₂⁺ center, it seems likely that Cys-95 is the residue accessible for labeling by 1,5-I-AEDANS. It was important to use highly purified adrenodoxin, since all 5 cysteines in denatured adrenodoxin have a free sulphydryl group available for modification by 1,5-I-AE-
DANS. AEDANS-adrenodoxin was found to have the same adrenodoxin reductase activity as the native adrenodoxin control, measured under conditions that are sensitive to the binding interaction between adrenodoxin and adrenodoxin reductase (Geren et al., 1983). This is in agreement with the studies of Miura et al. (1979) in which labeling of the single free cysteine on adrenodoxin with N-(1-Oxyl-2,2,6,6-tetramethyl-4-piperidinyl)maleimide showed no change in activity. Furthermore, the dissociation constant of the complex between AEDANS-adrenodoxin and cytochrome P-450 was found to be the same as that for native adrenodoxin (Fig. 2, Table II).

Fluorescence Energy Transfer Studies—The fluorescence spectrum of AEDANS-adrenodoxin was similar to that of other AEDANS-labeled proteins, with an excitation maximum at 340 nm and an emission maximum at 492 nm (Fig. 3). The quantum yield for AEDANS-adrenodoxin was found to be 0.50, using quinine bisulfate as a standard (Chen, 1967). Addition of cytochrome c, adrenodoxin reductase or cytochrome P-450,,, showed no change in activity.

Fluorescence quenching was most likely caused by Förster energy transfer from the AEDANS group to the heme or flavin groups, since the excitation and emission curves for AEDANS fluorescence were not affected by complex formation. Hudson and Weber (1973) have shown that environmental factors that change the quantum yield of AEDANS also cause a significant change on the wavelength for the maximum in the emission spectrum. The Förster energy transfer efficiency $E = 1 - F_{A} / F_{0}$ is inversely proportional to the sixth power of the distance $R$ between the donor and acceptor groups according to the relation (Stryer, 1978):

$$E = R^{6}/(R^{6} + R_{0}^{6})$$  \hspace{1cm} (2)

$R_{0}$ is defined as the distance at which the energy transfer efficiency is 50%, and is given by

$$R_{0} = (J_{A} \cdot Q_{o} n^{-4})^{1/6} \times 9.7 \times 10^{3}$$  \hspace{1cm} (3)

where $k^{2}$ is the orientation factor, $Q_{o}$ is the fluorescence quantum yield of the energy donor in the absence of acceptor, $n$ is the refractive index of the medium between the donor and acceptor, and $J$ is the spectral overlap integral:

$$J = \int F_{A} \lambda \, d\lambda / \int F_{D} \, d\lambda$$  \hspace{1cm} (4)

$F_{A}$ is the fluorescence of the donor in arbitrary units, the wavelength $\lambda$ is in centimeters, and $\alpha$ is the extinction coefficient of the acceptor in $M^{-1} \text{cm}^{-1}$.

The greatest source of uncertainty in $R_{o}$ is the orientation factor, which may in principle range from 0 to 4. However, if the donor and acceptor chromophores have polarizations less than the maximum of 0.5 due to local rotational diffusion or mixed transition dipole moments, then the uncertainty in $R_{o}$

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**Table I**

| Amino acid sequence of beef adrenodoxin (Tanaka et al., 1973; Okamura et al., 1985) |
|----------------------------------|--------|--------|--------|--------|--------|--------|--------|--------|
| Ser | Ser | Ser | Glu | Asp | Lys | Ile | Thr | Val | His | Phe | Ile | Asn | Arg | Asp | Gly | Thr | Leu | Thr |  |
| 10  | 20  | 30  | 40  | 50  | 60  | 70  | 80  | 90  | 100 | 110 | 120 |
| Thr | Lys | Gly | Lys | Ile | Gly | Asp | Ser | Leu | Leu | Asp | Val | Val | Val | Gln | Asn | Asn | Leu | Asp | Ile |
| 30  | 40  | 50  | 60  | 70  | 80  | 90  | 100 | 110 | 120 |
| Asp | Gly | Phe | Gly | Ala | Cys | Glu | Gly | Thr | Leu | Ala | Cys | Ser | Thr | Cys | His | Leu | Ile | Phe | Glu |
| 10  | 20  | 30  | 40  | 50  | 60  | 70  | 80  | 90  | 100 | 110 | 120 |
| Gln | His | Ile | Phe | Glu | Leu | Ala | Ile | Thr | Asp | Glu | Asn | Asp | Met | Leu | Asp | Leu | Ala | Met | |
| 10  | 20  | 30  | 40  | 50  | 60  | 70  | 80  | 90  | 100 | 110 | 120 |
| Ala | Tyr | Gly | Leu | Thr | Asp | Arg | Ser | Arg | Leu | Gly | Cys | Gln | Ile | Cys | Leu | Thr | Lys | Ala | Met | |
| 0  | 10  | 20  | 30  | 40  | 50  | 60  | 70  | 80  | 90  | 100 | 110 | 120 |
| Asp | Asn | Met | Thr | Val | Arg | Val | Pro | Asp | Ala | Val | Ser | Asp | Ala | Arg | Glu | Asp | Ile | Asp | Met | |
| 10  | 20  | 30  | 40  | 50  | 60  | 70  | 80  | 90  | 100 | 110 | 120 |
| Gly | Met | Asn | Ser | Ser | Lys | Ile | Glu |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
Interaction of Adrenodoxin with Cytochrome P-450

The interaction of cytochrome P-450 with native adrenodoxin (●), maleylated adrenodoxin (●), or AEDANS-adrenodoxin (□) was determined by following the conversion of low spin ferric heme to high spin using \( \Delta A = A_{428} - A_{450} \). The solution contained 20 mM Hepes, pH 7.2, 50 mM NaCl, 0.1 mM EDTA, 0.1% Tween 80, 0.1 mM dithiothreitol, 26 \( \mu \)M cholesterol, and 0.42 \( \mu \)M cytochrome P-450. The reference cuvette contained the same concentration of adrenodoxin as the sample cuvette. The EDTA, 0.1% Tween 80, 0.1 mM dithiothreitol, 26 \( \mu \)M cholesterol, and adrenodoxin 0.1% Tween 80, pH 7.2, 0.1 mM DTT, 0.1 mM EDTA, 0.1% Tween 80, and 50 mM sodium chloride.

The solution contained 20 mM Hepes, pH 7.2, 0.1 mM DTT, 0.1 mM EDTA, 0.1% Tween 80, 0.42 \( \mu \)M cytochrome P-450, and adrenodoxin reductase, and cytochrome P-450, was determined by following the conversion of low spin ferric heme to high spin using \( \Delta A = A_{428} - A_{450} \). The solution contained 20 mM Hepes, pH 7.2, 50 mM NaCl, 0.1 mM EDTA, 0.1% Tween 80, 0.1 mM dithiothreitol, 26 \( \mu \)M cholesterol, and 0.42 \( \mu \)M cytochrome P-450. The reference cuvette contained the same concentration of adrenodoxin as the sample cuvette. The solid line is a nonlinear regression fit of equation 1 (with \( 1 = \Delta A/\Delta A_s \) substituted for \( 1 \)) to the experimental data.

**Table II**

Dissociation constants for the complex between cytochrome P-450, and adrenodoxin derivatives

<table>
<thead>
<tr>
<th>Derivative</th>
<th>( K_d ) (( \mu M ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native adrenodoxin</td>
<td>0.15 ± 0.01</td>
</tr>
<tr>
<td>Maleyl-adrenodoxin</td>
<td>0.15 ± 0.01</td>
</tr>
<tr>
<td>AEDANS-adrenodoxin</td>
<td>0.13 ± 0.01</td>
</tr>
<tr>
<td>p-Hydroxyphenylglyoxal-adrenodoxin</td>
<td>0.11 ± 0.01</td>
</tr>
<tr>
<td>Diethylpyrocarbonate-adrenodoxin</td>
<td>0.40 ± 0.10</td>
</tr>
</tbody>
</table>

**FIG. 2.** The interaction of cytochrome P-450, with native adrenodoxin (●), maleylated adrenodoxin (●), or AEDANS-adrenodoxin (□) was determined by following the conversion of low spin ferric heme to high spin using \( \Delta A = A_{428} - A_{450} \). The solution contained 20 mM Hepes, pH 7.2, 50 mM NaCl, 0.1 mM EDTA, 0.1% Tween 80, 0.1 mM dithiothreitol, 26 \( \mu \)M cholesterol, and 0.42 \( \mu \)M cytochrome P-450. The reference cuvette contained the same concentration of adrenodoxin as the sample cuvette. The solid line is a nonlinear regression fit of equation 1 (with \( 1 = \Delta A/\Delta A_s \) substituted for \( 1 \)) to the experimental data.

**TABLE III**

Fluorescence energy transfer parameters for the complexes of AEDANS-adrenodoxin with adrenodoxin reductase, cytochrome P-450, and cytochrome c

<table>
<thead>
<tr>
<th>Complex</th>
<th>( K_d ) (( \mu M ))</th>
<th>( J )</th>
<th>( E )</th>
<th>( R_o ) (( \AA ))</th>
<th>( R ) (( \AA ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome c</td>
<td>0.09 ( 6.3 \times 10^{-14} )</td>
<td>40 ± 4</td>
<td>41</td>
<td>42 ± 2</td>
<td></td>
</tr>
<tr>
<td>Adrenodoxin reductase</td>
<td>0.07 ( 2.2 \times 10^{-14} )</td>
<td>42 ± 3</td>
<td>34</td>
<td>36 ± 2</td>
<td></td>
</tr>
<tr>
<td>Cytochrome P-450,</td>
<td>0.01 ( 1.4 \times 10^{-14} )</td>
<td>21 ± 5</td>
<td>47</td>
<td>58 ± 4.0</td>
<td></td>
</tr>
</tbody>
</table>

**FIG. 3.** Fluorescence quenching of AEDANS-adrenodoxin by cytochrome P-450, adrenodoxin reductase, and cytochrome c. The buffer was 5 mM MOPS, pH 7.5, for adrenodoxin reductase and cytochrome c, and 20 mM HEPES, pH 7.2, 0.1 mM EDTA, 0.1 mM dithiothreitol, and 26 \( \mu \)M cholesterol for cytochrome P-450. The solutions contained 0.42 \( \mu \)M AEDANS-adrenodoxin and 2.7 \( \mu \)M cytochrome P-450, 3 \( \mu \)M adrenodoxin reductase, or 3.4 \( \mu \)M cytochrome c. The excitation wavelength was 340 nm, and the fluorescence scale is in arbitrary units.

**FIG. 4.** Fluorescence quenching of AEDANS-adrenodoxin by cytochrome P-450, adrenodoxin reductase, or cytochrome c (●). The conditions were the same as those in Fig. 3, and an emission wavelength of 480 nm was used. The solid lines are nonlinear regression fits of Equation 1 to the experimental data.

The fluorescence polarization for the complexes with adrenodoxin reductase and cytochrome P-450, were found to be 0.0, indicating that the local rotational diffusion of the AEDANS label was fast compared to the fluorescence lifetime. The overall rotational diffusion of these complexes is too slow, relative to the fluorescence lifetime (16 ns), to affect the polarization. Under these conditions the orientation factor has a value of \( V_3 \). Using the spectral overlap integrals and other parameters given in Table III, the fluorescence energy transfer measurements indicated that the AEDANS group on adrenodoxin Cys-95 was 42 Å from the heme group in cytochrome c, 36 Å from the FAD group in adrenodoxin reductase, and 58 Å from the heme group in cytochrome P-450.

The fluorescence of AEDANS-adrenodoxin was not detectably quenched by anaerobic addition of reduced adrenodoxin reductase (data not shown). This is reasonable, since the absorption spectrum of reduced FADH₂ does not overlap with the fluorescence of AEDANS. There was also no significant quenching by addition of reduced cytochrome P-450 or its CO complex, suggesting that these compounds do not form a complex with adrenodoxin, an observation in agreement with the results of Tsubaki (1986) using resonance Raman spectroscopy.

Modification of Lysine, Arginine, and Histidine Residues on Adrenodoxin—Treatment with maleic anhydride modified over 98% of the lysine residues on adrenodoxin to form negatively charged maleylated amino groups. This modification did not affect the UV visible spectrum of adrenodoxin, indicating that the iron-sulfur center remained intact. There was also no change in adrenodoxin reductase activity or the dissociation constant of the complex with cytochrome P-450.
(Fig. 2, Table II). Complete modification of all the arginine residues with p-hydroxyphenylglyoxal did not affect the UV visible spectrum of adrenodoxin or the adrenodoxin reductase activity as previously reported by Geren et al. (1986). There was also no change in the dissociation constant of the complex with cytochrome P-450<sub>sec</sub> (Table II). Histidine modification was carried out using diethylpyrocarbonate as described by Miles (1977). This treatment can result in either single or double modification of histidine, the latter involving opening of the imidazole ring. Modification of 100% of the three histidine residues did not affect the UV visible spectrum or the adrenodoxin reductase activity of adrenodoxin, confirming earlier studies using assay conditions sensitive to both $K_m$ and $V_{max}$ (Geren et al., 1986). However, the dissociation constant of the complex with cytochrome P-450<sub>sec</sub> was increased 3-fold compared to that of native adrenodoxin (Table II).

**DISCUSSION**

Fluorescence energy transfer techniques have been widely used to estimate distances within protein complexes. In the present study we have developed conditions to specifically label adrenodoxin Cys-95 with 1,5-I-AEDANS. The other 4 cysteine residues in adrenodoxin are liganded to the Fe<sub>B8</sub>S<sub>B</sub> center and are not available for 1,5-I-AEDANS labeling. The fluorescence of AEDANS-adrenodoxin was significantly quenched by complex formation with cytochrome c or adrenodoxin reductase and to a smaller extent with cytochrome P-450<sub>sec</sub>. Fluorescence energy transfer calculations indicated that the AEDANS group on adrenodoxin Cys-95 was 42 Å from the heme group in cytochrome c, 36 Å from the FAD group in adrenodoxin reductase, and 58 Å from the heme group in cytochrome P-450<sub>sec</sub>. In discussing these results, it is useful to consider a model for the structure of adrenodoxin that is based on an alignment of its sequence with that of *Spirulina platensis* ferredoxin, for which an x-ray crystal structure has been determined (Fukuyama et al., 1980). Although there are significant differences between these two sequences, the highly conserved ferredoxin sequence 40–49 surrounding the iron-sulfur “cluster cavity” aligns well with adrenodoxin residues 45–55. In addition, the ferredoxin sequence 59–77 contains 6 acidic residues that align well with acidic residues in the adrenodoxin sequence 65–84, and a semi-invariant Tyr-75 that corresponds to adrenodoxin Tyr-82. This sequence forms a loop at the surface of the protein with a β turn at residues 66–69 that corresponds to the predicted β turn at residues 72–75 of adrenodoxin (Bicknell-Brown et al., 1981). We have recently used a water-soluble carbodiimide to determine that the carbohydrate groups at adrenodoxin residues 72, 73, 74, 76, 79, and 86 in this highly acidic sequence are involved in binding adrenodoxin reductase, cytochrome P-450<sub>sec</sub>, and cytochrome c (Geren et al., 1984; Lambeth et al., 1985). In the above model, Cys-95 is located on the opposite side of adrenodoxin from this acidic binding domain, and about 32 Å distant from it. This is consistent with the fluorescence energy transfer data for the complex with cytochrome c. We have previously shown that this complex involves electrostatic interactions between carbohydrate groups at the acidic domain on adrenodoxin and lysine amino groups surrounding the heme crevice of cytochrome c (Geren and Millett, 1981b). Since the center of the heme group is located 9 Å beneath the binding surface of cytochrome c (Dickerson, 1975), the postulated distance between Cys-95 on the “back” of adrenodoxin and the heme group of cytochrome c would be about 32 Å + 9 Å = 41 Å, in agreement with the fluorescence energy transfer measure-

ment. If it is assumed that adrenodoxin reductase binds to the same acidic domain on adrenodoxin as cytochrome c does, then the fluorescence energy transfer measurements indicate that the FAD group in adrenodoxin reductase would be located only about 4 Å (36 Å - 32 Å) beneath the binding surface for adrenodoxin. If the same assumption is made for the complex between adrenodoxin and cytochrome P-450<sub>sec</sub>, then the heme group would be located 26 Å (58 Å - 32 Å) from the binding interface between the two proteins. This indicates that the heme group in cytochrome P-450<sub>sec</sub> is deeply buried 26 Å beneath the surface where adrenodoxin binds. X-ray crystallographic studies have revealed that the heme group in the analogous protein cytochrome P-450<sub>s</sub> is deeply buried within the protein 15 Å from the nearest surface and nearly 30 Å from the other surfaces (Poulos et al., 1985). It is not known, however, where the binding domain for putidaredoxin is located.

Usanov et al. (1985) and Chashchin et al. (1985) have recently proposed that there are distinct binding sites on adrenodoxin for adrenodoxin reductase and cytochrome P-450<sub>sec</sub> and that electron transfer occurs in an organized trimeric complex. This model is based on the finding that a cross-linked complex of adrenodoxin and adrenodoxin reductase is able to transfer electrons to cytochrome P-450<sub>sec</sub>, and a cross-linked complex of adrenodoxin and cytochrome P-450<sub>sec</sub> is able to accept electrons from adrenodoxin reductase. However, in both of these studies the reagents N-succinimidyl-3-(2-pyridyldithio)propionate and methyl-4-mercaptobutyrimidate were used to form a 13 Å long cross-link between a lysine on adrenodoxin and a lysine on the partner. This length is more than sufficient to allow the cross-linked adrenodoxin enough rotational flexibility to transfer electrons from the reductase to the P-450. Furthermore, our finding that complete maleylation of all the lysines on adrenodoxin did not affect its interaction with either adrenodoxin reductase or cytochrome P-450<sub>sec</sub> indicates that there are no lysine residues located at the binding site on adrenodoxin. Thus, the cross-link used in the above studies is attached to a remote site on adrenodoxin. In contrast, when the water-soluble carbodiimide EDC was used to form “zero-length” cross-links between carboxyl groups on adrenodoxin and lysine amino groups on adrenodoxin reductase, the complex was not able to transfer electrons to cytochrome P-450<sub>sec</sub> (Usanov et al., 1985). This finding is consistent with our proposal that carboxyl groups in the highly acidic adrenodoxin sequence 73–86 are involved in binding both adrenodoxin reductase and cytochrome P-450<sub>sec</sub>. Cytochrome c also appears to bind to this same domain on adrenodoxin, since an EDC-cross-linked adrenodoxin-cytochrome c complex was unable to accept electrons from adrenodoxin reductase (Geren et al., 1984).

It was also found that complete modification of all the arginine residues on adrenodoxin with p-hydroxyphenylglyoxal did not affect the interaction with adrenodoxin reductase or cytochrome P-450<sub>sec</sub>. Since this reagent is quite bulky, it is unlikely that any arginine residues are located at the binding site. It should be noted however, that there is an arginine at residue 87 at the edge of the proposed binding domain. The only chemical modification that had a differential effect on the activities of adrenodoxin was that using diethylpyrocarbonate, which can result in either single or double modification of histidine (Miles, 1977). Modification of adrenodoxin with 3 eq of diethylpyrocarbonate had no effect on the activity with adrenodoxin reductase but increased the dissociation constant of the complex with cytochrome P-450<sub>sec</sub> by 3-fold. However, it could not be determined how these 3 eq were distributed among the 3 histidine
residues, since double modification of 1 histidine results in the same spectral change as single modification of 2 histidines. Two of the histidine residues on adrenodoxin are well removed from the proposed binding domain, but the highly conserved His-56 is adjacent to Cys-55 which ligands the iron-sulfur center. It is possible that modification of this residue leads to a conformational change that differentially affects the interaction with the reductase and the P-450. Double modification of all 3 histidines in adrenodoxin leads to removal of the iron-sulfur center and loss of all enzymatic activity (Geren et al., 1986).

REFERENCES