Complex Formation between Flavodoxin and Cytochrome c

CROSS-LINKING STUDIES

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Complex formation between Azotobacter vinelandii flavodoxin and horse cytochrome c has been demonstrated through cross-linking studies with dimethyl suberimidate, dimethyl adipimidate, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, and dimethyl-3,3′-dithiobispropionimidate. Essentially quantitative cross-linking of cytochrome c and flavodoxin was observed at low ionic strengths with the carbodiimide cross-linking reagent. An association constant of $4 \times 10^4 \text{ M}^{-1}$ was obtained between cytochrome c and flavodoxin at 88 mM ionic strength from analysis of the cross-linking studies. This value is similar to the association constant determined kinetically during the electron transfer reaction between cytochrome c and flavodoxin (Simonsen, R. P., Weber, P. C., Sulemme, F. R., and Tollin, G. (1982) Biochemistry 21, 6366–6375), and suggests that the cross-linked complex may be similar to the precursor complex identified kinetically. A structural model for the flavodoxin-cytochrome c complex proposed by these workers is shown to be compatible with the present cross-linking results.

A recent kinetic study of the reduction of cytochrome c by the semiquinone form of flavodoxin demonstrated that the electron transfer rate decreased with increasing ionic strength, while saturation kinetics were observed at low ionic strength (1). These results were interpreted as providing evidence for an electrostatically stabilized complex between the positively charged cytochrome and negatively charged flavodoxin molecules. At low ionic strengths, the dissociation constant for the complex was calculated to be approximately 10 μM. Using a computer graphics procedure which matched complementary charged regions on the two proteins (2), a model for this intermediate was derived from the known structures and sequences of cytochrome c (3) and flavodoxin (4). This technique had previously been used to generate possible structures for the electrostatically stabilized complexes of cytochrome c with cytochrome b₅ (2) and cytochrome c peroxidase (5). These modeling studies have suggested that complex formation facilitates electron transfer between two proteins by maintaining the redox groups in a favorable orientation (6, 7).

No direct evidence for complex formation between cytochrome c and flavodoxin was obtained in the study of Siemann et al. (1), however. As recognized by these authors, alternate mechanisms could also give apparent saturation kinetics, such as formation of a nonproductive complex or the involvement of an activated form of one of the redox partners. In an attempt to demonstrate formation of a cytochrome c-flavodoxin complex, we initiated a series of cross-linking experiments. When successful, these studies provide positive identification of complex formation, since two proteins will be covalently linked only if suitable residues on each molecule are sufficiently close to react with the same bifunctional reagent. Cross-linking studies have previously been used to study complex formation between cytochrome c and cytochrome c oxidase (8), cytochrome c peroxidase (9), plastocyanin (10), reaction centers from Rhodopseudomonas sphaeroides (11), and adrenodoxin (12). As described below, we have detected formation of a flavodoxin-cytochrome c complex using both noncleavable and cleavable cross-linking reagents. These results are related to the structural predictions of the proposed model for the complex.

MATERIALS AND METHODS

Flavodoxin was purified from Azotobacter vinelandii by a modification of the procedure of Hinkson and Bulen (13). Horse cytochrome c, dimethyl suberimidate (DMS²), dimethyl adipimidate (DMA), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), dimethyl-3,3′-dithiobispropionimidate (DTBP), and molecular weight standards for gel electrophoresis were obtained from Sigma and used without further purification. Concentrations of proteins were determined using published extinction coefficients of $ε_{600} = 108 \text{ M}^{-1} \text{cm}^{-1}$ for oxidized cytochrome c (14), and $ε_{600} = 106 \text{ M}^{-1} \text{cm}^{-1}$ for oxidized flavodoxin (15).

Noncleavable Cross-linking—Experiments with the noncleavable cross-linking agents DMS and DMA were performed as described by Davies and Stark (15). Solutions containing 16–75 μM oxidized cytochrome c and 35–120 μM oxidized flavodoxin were incubated in 0.2 M triethanolamine-HCl, pH 8.5, with either 1 mg/ml DMS or 1 mg/ml DMA at 20 °C. Aliquots were taken at various times and quenched for 10 min after addition of ammonium acetate to 10 mM concentration. The samples were then heated to 70 °C for 10 min with 1% SDS and 2% β-mercaptoethanol, and analyzed by SDS-polyacrylamide slab gel electrophoresis (16). Acrylamide concentrations of 13% and 3% were used for the lower and stacking gels, respectively. Protein bands were visualized by staining with Coomassie Blue R-250 and destaining in 10% acetic acid. Gel scans were recorded with a Helena Quik-Scan densitometer.

The experimental protocol for cross-linking with EDC was based on previously described procedures (8–10, 12). Solutions containing 20 μM oxidized flavodoxin and 20–250 μM oxidized cytochrome c were incubated with 2 mM EDC in 5 mM MOPS, pH 7.2, at 20 °C. The ionic strength of the solution was adjusted by addition of NaCl. After 12 h, products were analyzed by SDS-gel electrophoresis as described above.

The abbreviations used were: DMS, dimethyl suberimidate; DMA, dimethyl adipimidate; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; DTBP, dimethyl-3,3′-dithiobispropionimidate; SDS, sodium dodecyl sulfate; MOPS, 3-(N-morpholino)propanesulfonic acid.

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Cleavable Cross-linking—Cross-linking with the cleavable reagent DTBP was performed as described by Wang and Richards (17, 18). Solutions containing 45–338 μM oxidized cytochrome c and 100 μM flavodoxin in 0.2 M triethanolamine-HCl, pH 8.5, were incubated with 1 mg/ml DTBP for 3 h at room temperature. The cross-linking reaction was quenched by addition of ammonium acetate to 10 mM concentration. After 10 min, SDS and n-ethylmaleimide were added to final concentrations of 2% and 1 mg/ml, respectively. Each sample was then heated at 70 °C for 20 min, in the absence of β-mercaptoethanol.

Two-dimensional gel electrophoresis was performed following a protocol similar to that of Wang and Richards (17, 18). The first dimension was run on a 1.5-mm slab gel, with the same acrylamide concentrations as described above. After running the gel until the tracking dye reached the bottom of the slab, a 10-mm wide strip of each sample was cut from the gel. The diethylbridge in DTBP was cleaved prior to running the second dimension by incubating the gel strip in 10% β-mercaptoethanol, 0.125 M tri-HCl, pH 8.8, 0.1% SDS for 2 h. The strip was then secured with 1% agarose to the top of a second slab gel of 3.0-mm thickness. After running the gel in the second dimension, protein bands were visualized by the staining procedure described above.

Estimation of Association Constants from Cross-linking Data—The yield of cross-linked complex will depend on the association constant between cytochrome c and flavodoxin, the rate of the cross-linking reaction, and the rate of inactivation of the cross-linking reagent by side reactions. Association constants between flavodoxin and cytochrome c were estimated from kinetic studies (1) to be on the order of 10¹⁰ M⁻¹, depending on the ionic strength. If the cross-linked complex corresponds to the kinetically detected complex, similar association constants should be obtained from the cross-linking studies.

The following mechanistic scheme was employed to extract flavodoxin-cytochrome c association constants from the cross-linking studies:

\[
\begin{align*}
F + C & \xrightarrow{K} FC \\
& \xrightarrow{k(R)} X \\
& \xrightarrow{R} I
\end{align*}
\]

where \( F \), \( C \), \( FC \), \( X \), and \( I \) represent flavodoxin, cytochrome c, flavodoxin-cytochrome c complex, cross-linked flavodoxin-cytochrome c complex, cross-link reagent, and inactive cross-link reagent, respectively. \( K \), \( k \), and \( k_r \) are the flavodoxin-cytochrome c association constant, cross-linking rate constant and the cross-linker inactivation rate constant, respectively. This scheme neglects reactions of \( R \) with either protein which do not result in cross-link formation.

From this model, the rates of change of \((X)\) and \((R)\) are given by the expressions:

\[
\begin{align*}
\frac{d(X)}{dt} &= k(R)(FC) - kX \\
\frac{d(R)}{dt} &= -h(R) - kR(F)(C)
\end{align*}
\]

Let \( F_R \), \( C_R \), \( B_R \), and \( X_R \) be the total concentration of flavodoxin, cytochrome c, cross-link reagent, and cross-linked species, respectively. Under experimental conditions where \( R_B \gg R_F \), Equation 2 may be reduced to:

\[
\left[\frac{k(1 + KC_R)}{kC_R(F_R - X)} + 1\right]d(X) = -d(R)
\]

Using the relationships that \((X) = 0\) and \((R) = R_F\) at the beginning of the reaction, and \((X) = X_R\) and \((R) = 0\) at the completion of the reaction, Equation 3 may be integrated to give:

\[
\ln\left[\frac{F_R}{X_R}\right] = \frac{KCC_R}{k(1 + KC_R)}(R_F - X_F)
\]

Since \( X_F \) cannot exceed \( F_R \), under the experimental restrictions described above \((R_F \gg R_B)\), \( X_R \approx X_F \) may be approximated by \( R_F \). With this substitution, Equation 4 may be rearranged to give:

\[
\ln f = \frac{kR_F}{k} + \frac{\ln f}{KC_R}
\]

where \( f = (F_R - X_R)/F_R \) equals the fraction of flavodoxin not cross-linked. This equation has a form similar to the Scatchard equation used for the analysis of equilibrium binding data.

According to Equation 5, the flavodoxin-cytochrome c association constant may be obtained from the reciprocal of the slope of a plot of \( -\ln f \) versus \( -\ln f/HC_R \). This association constant will presumably represent an underestimate to the true value, since all side reactions between proteins and \( R \) which do not lead to cross-link formation will increase the rate of inactivation of \( R \), and consequently decrease the yield of cross-linked products.

Computer Modeling—Atomic coordinates for the oxidized form of Clostridium MP flavodoxin and the reduced form of tuna cytochrome c were obtained from the Brookhaven Protein Data Bank (19) (coordinate sets 3FXN and 4CYT, respectively). Coordinates for the proposed flavodoxin-cytochrome c complex were generated by a non-linear least squares fit of the α-carbon coordinates for flavodoxin residues 7, 8, 9, 18, 63, 64, 65, and 120 and cytochrome c residues 13, 14, 15, 16, 17, 25, 77, 79, and heme iron to the positions depicted in Fig. 8 of Simonsen et al. (1). Rigid body rotation and translation parameters were refined in this procedure. Intermolecular separation distances reported in Ref. 1 were reproduced to within 0.2 Å by this procedure. No further adjustments (such as repositioning side chain atoms) were made to the atomic positions in the model.

The sequence alignment proposed by Dubord and King Fox (20) was used to correlate residues in the Azotobacter flavodoxin with the clostridial flavodoxin used in the crystallographic work. For the calculations described below, we have assumed that the positions of the main chain atoms for homologous residues are identical in the two proteins. Accordingly, the α-carbon positions for lysines 2, 13, 16, 18, 22, 23, 50, 55, 115, 125, and 150 of the Azotobacter flavodoxin were taken from the coordinates for residues 2, 11, 14, 17, 20, 21, 72, 80, 104, 109, and 123 of the clostridial flavodoxin. No equivalent residue was indicated for lysine 145 of Azotobacter flavodoxin. A similar procedure was used to obtain the coordinates for the lysine residues in horse cytochrome c, based on the tuna cytochrome c structure.

The reaction between the bifunctional cross-linking reagents DMS or DMS and 2 lysine residues generates a structure containing 18 or 20 atoms, respectively, between the two α-carbon atoms. In a fully extended conformation, these chains would span approximately 20–22 Å. This distance range provides an upper limit for the α-carbon separation between two cross-linked lysine residues.

RESULTS AND DISCUSSION

The reactions of EDC, DMS, DMS, and DTBP with flavodoxin and cytochrome c result in the appearance of a major new band, as visualized by SDS-gel electrophoresis. Gel scans of cross-linking experiments with EDC at varying ionic strengths are illustrated in Fig. 1. Based on the following considerations, the cross-linked species appears to contain one molecule each of flavodoxin and cytochrome c. (i) At low ionic strength, both proteins can be almost quantitatively incorporated into the cross-linked species, when initially present in equimolar ratios (Fig. 1a). This behavior requires that the complex contain equal amounts of the two proteins. At higher ionic strengths, however, the cross-linking reaction proceeds with lower yields. This suggests that the complex between cytochrome c and flavodoxin is stabilized by complementary interactions, the strength of which decreases with increasing ionic strength. (ii) From the mobility of the cross-linked band on the gels, an apparent molecular weight of 35,000 is estimated. This value is approximately the sum of the molecular weights of flavodoxin (20,000) and cytochrome c (13,500). (iii) The presence of both proteins in the cross-linked species was directly demonstrated through the use of the cleavable cross-linking reagent DTBP. Reaction of cytochrome c with DTBP (as well as both DMS and DMS) leads to formation of the cross-linked species, although with approximately 20% of the yield observed with EDC. Two-dimensional analysis of the reduction products from the DTBP cross-linking reaction (Fig. 2) demonstrates that the cross-
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Fig. 1. SDS-polyacrylamide gel electrophoresis of flavodoxin (fld) cross-linked to cytochrome c (cyt c) by EDC. Samples contained 45 μM cytochrome c and 50 μM flavodoxin. Gel scans labeled a, b, c, and d represent product analysis of cross-linking experiments at ionic strengths of 5, 88, 170, and 340 mM, respectively. The splitting of the flavodoxin peak observed at higher ionic strengths is apparently due to the formation of an internal cross-link. Since the mobility of native flavodoxin corresponds to the slower moving band, it appears that formation of the internal cross-link is favored at lower ionic strengths.

linked species (band b) contains both cytochrome c and flavodoxin. At the protein concentrations used in this particular experiment, significant amounts of other cross-linked products were also produced (such as bands a and c). Control experiments identified band a as a cytochrome c dimer, while band c consisted of both cytochrome c trimers and flavodoxin dimers. Cleavage of the high molecular weight cross-linking products indicated that both cytochrome c and flavodoxin are present in these species. Since no discrete bands were evident in this region, it seems possible that these bands are due to higher, random aggregates of cross-linked proteins. Similar results were observed during cross-linking of cytochrome c to the photosynthetic reaction centers from R. sphaeroides (11).

Fig. 2. Two-dimensional SDS-polyacrylamide gel electrophoresis analysis of flavodoxin and cytochrome c cross-linked with DTBP. The cross-linked sample contained 230 μM cytochrome c and 100 μM flavodoxin. The second dimension was run after the cross-link was cleaved with β-mercaptoethanol. Band b cleaved to release both cytochrome c and flavodoxin. Band a corresponds to a cytochrome c dimer, while band c consists primarily of flavodoxin dimer, although some cytochrome c trimer is present (the cleavage products include both cytochrome c dimer and monomer species).

Association constants between cytochrome c and flavodoxin were obtained from the EDC cross-linking experiments using the analysis described under "Materials and Methods." The fraction, f, of flavodoxin remaining uncross-linked was monitored as a function of total cytochrome c concentration. Plots of −ln f versus −ln fc are illustrated in Fig. 3 for ionic strengths of 88 and 170 mM. Equation 5 predicts that these plots should be linear, with slope −1/K and intercept kRf/kc. Since the intercept should be independent of ionic strength and protein concentration, the least squares lines plotted in Fig. 3 were constrained to have identical intercepts. Association constants of (4.0 ± 1.5) × 10^5 M^-1 and (5.2 ± 0.8) × 10^5 M^-1 were obtained for ionic strengths of 88 and 170 mM, respectively, with an intercept value of 1.8 ± 0.2. If the two curves were allowed to have independent intercepts, association constants of (3.1 ± 0.8) × 10^5 M^-1 and (8.3 ± 0.9) × 10^5 M^-1 for the lower and higher ionic strengths, respectively, were obtained.

From analysis of the kinetics of electron transfer, Simonsen et al. (1) estimated the flavodoxin-cytochrome c association constant to be 18 × 10^5 M^-1 at 80 mM ionic strength. This value is approximately five times higher than the value obtained from the cross-linking studies at 88 mM ionic strength. The agreement between the two values is reasonable, however, since the previously discussed limitations of the cross-linking analysis will probably underestimate the true association constant. Although the kinetic studies could not detect complex formation at ionic strengths above 80 mM, the cross-linking results indicate that complex formation still occurs, although with reduced association constants.

As described under "Materials and Methods," observation of cross-linking between flavodoxin and cytochrome c with DMA, DMS, and DTBP imposes an upper limit on the separation distance between lysine residues in the two proteins. A simple test of the model of the complex proposed by
In the proposed model for the complex, lysine 13 of cytochrome c participates directly in the binding interactions with flavodoxin. Consequently, this residue may not be readily accessible for cross-linking. Lysine 8 is not included in the interface region, however, and may represent the principal cross-linking site.

Since the site of cross-linking between cytochrome c and flavodoxin is proposed to involve the amino-terminal region of each protein, it is possible that sequence analysis of the cross-linked protein may be used to directly establish the sites of interaction. A partial purification of the DMS cross-linked products was achieved with a mono-Q anion exchange column developed with 0.1–0.5 M NaCl gradient, 0.02 M Tris-HCl, pH 7.5 (data not shown). Sequence analysis of this sample on an Applied Biosciences Model 470 sequencer failed to release any amino acid residues, however. This suggests that DMS blocks the sequence analysis by reacting with the amino terminal nitrogen of flavodoxin (the amino group of cytochrome c is naturally blocked with an acetyl group). No further attempts were made to directly identify the site of cross-linking.

The ability to cross-link flavodoxin and cytochrome c demonstrates that a complex of the two proteins exists in solution. Similarities in the complex stoichiometry and association constants determined in the present work with the results of earlier kinetic studies (1) suggest that the cross-linked complex is similar to the kinetically significant complex. Covalent cross-linking of flavodoxin and cytochrome c will facilitate isolation and characterization of a stable analogue of a precursor complex to electron transfer. Of particular interest, it will now be possible to attempt the crystallization of this complex under a wide range of solvent conditions, with less concern about the stability of a freely dissociating complex under changing solvent conditions. Successful crystallization of a flavodoxin-cytochrome c species will ultimately allow the use of x-ray diffraction methods to directly describe structural features of the complex.

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