Interaction of Cytochrome *c* with Reaction Centers of *Rhodopseudomonas sphaeroides* R-26: Localization of the Binding Site by Chemical Cross-Linking and Immunochemical Studies†

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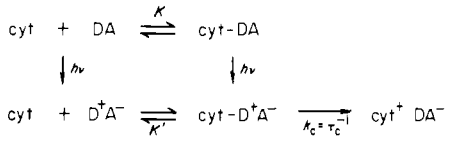
ABSTRACT: The location of the cytochrome binding site on the reaction center of *Rhodopseudomonas sphaeroides* was studied by two different approaches. In one, cross-linking agents, principally dithiobis(propionimidate) and dimethyl suberimidate, were used to link cytochrome *c* and cytochrome c_2 to reaction centers; in the other, the inhibition of electron transfer by antibodies against the subunits was investigated. Cytochrome *c* (horse) cross-linked to the L and M subunits, whereas cytochrome c_2 (*R. sphaeroides*) cross-linked only to the L subunit. The cross-linked reaction center–cytochrome complexes were isolated by affinity chromatography. The rate of electron transfer in the cross-linked cytochrome c_2 complex was the same as that in the un-cross-linked complex. However, when cytochrome *c* was used, the rate in the cross-linked complex was about 15 times slower than that in the un-cross-linked complex. Fab fragments of antibodies specific against the L and M subunits blocked electron transfer from both cytochrome *c* (horse) and cytochrome c_2 (*R. sphaeroides*). Antibodies specific for the H subunit did not block either reaction. We conclude that the cytochrome binding site on the reaction center is close (~10 Å) to both the L and M subunits, possibly in a cleft between them.

Bacterial photosynthesis involves the light-induced electron transfer from a primary donor (D),[1] a bacteriochlorophyll dimer, to a primary acceptor (A), a quinone–iron complex, in a membrane-bound bacteriochlorophyll–protein complex called the reaction center (RC) [e.g., see Feher & Okamura (1978)]. The RC–protein consists of three polypeptides, L, M, and H (light, medium, and heavy, respectively), one of which (H) is not required for the primary photochemistry. The oxidized primary donor is rapidly reduced in vivo by a cytochrome (cyt) molecule (Parson, 1968; Dutton & Prince, 1978). In *Rhodopseudomonas sphaeroides*, this cytochrome is a water-soluble cytochrome c_2 species (Bartsch, 1963, 1978).

The simplified reaction scheme of isolated RC with cytochrome can be schematically represented as follows:



At low salt and high cytochrome concentrations, a cytochrome–RC complex was shown to be formed (Ke et al., 1970; Overfield et al., 1979; Rosen et al., 1979). Under these conditions, a first-order rate constant ($k_c = 1/\tau_c^{-1}$) independent of cytochrome concentration was found for both cyt c_2 (τ_c = 1 μs) and mammalian cyt *c* (τ_c = 20 μs). Equilibrium dialysis measurements at low salt concentrations have shown that a 1:1 complex of the RC with cyt *c* and c_2 with a dissociation constant *K* (≃1 μM) is formed (Rosen et al., 1979, 1980).

In the present study, the location of the cytochrome binding site on the RC was investigated by the techniques of chemical cross-linking and immunochemical inhibition. Both cyt *c* from horse heart and cyt c_2 from *R. sphaeroides* were studied, the former more extensively than the latter. In the cross-linking studies, bifunctional reagents were used to cross-link functional groups of nearby amino acid residues (Wang & Richards, 1974; Das & Fox, 1979). If suitable amino acid residues (e.g., lysine) on the RC and cytochrome are near to each other (~10 Å) in the RC–cytochrome complex, cross-linking between cytochrome and RC will occur. Thus, cross-linking is capable of giving information about the location of the cytochrome with respect to the various RC subunits. The cross-linked complex was isolated and its photochemical activity compared to that of the un-cross-linked complex. In the immunochemical inhibition studies, antibodies (Ab) against the isolated subunits were prepared. Fab fragments of these antibodies were assayed for their ability to block fast electron transfer between RCs and cytochrome. If the antibody is bound near the cytochrome binding site, an inhibition of the electron transfer reaction between cytochrome and RCs is expected and indeed was observed. A preliminary account of this study has been presented earlier (Rosen et al., 1979).

Experimental Procedures

Materials. Reaction centers were prepared by using the detergent LDAO as previously described (Feher & Okamura, 1978), and their concentration was determined from the extinction coefficient, $\epsilon_{802}^{1cm} = 288$ mM^{-1} cm^{-1} (Straley et al., 1973). Horse heart cyt *c* (type III) was obtained from Sigma Chemical Co.; cyt c_2 was isolated from the *R. sphaeroides* as described by Bartsch (1978). The concentration of cytochrome was obtained from the extinction coefficients, ϵ_{550}^{1cm} (reduced) of 27.6 and 30.8 mM^{-1} cm^{-1} for cyt *c* and c_2, respectively

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[1] Abbreviations: RC, reaction center; DTBP, dithiobis(propionimidate); DMS, dimethyl suberimidate; DMA, dimethyl adipimidate; D, primary donor; A, primary acceptor; cyt, cytochrome; Ab, antibody; Fab, antibody fragment after papain digestion; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*'-2-ethanesulfonic acid; LDAO, lauryldimethylamine oxide; $NaDodSO_4$, sodium dodecyl sulfate; DTT, dithiothreitol; Az, ethyl [(5-azido-2-nitrobenzoyl)amino]acetimidate; Tris, tris(hydroxymethyl)-aminomethane; Q-0, 2,3-dimethoxy-5-methyl-1,4-benzoquinone.

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(Margoliash & Frohwirt, 1959; Bartsch, 1978). The cytochrome content of the RC–cytochrome complex was determined by the pyridine hemochromogen assay (Bartsch, 1963). The cross-linking reagents, dithiobis(propionimidate) (DTBP), dimethyl suberimidate (DMS), and dimethyl adipimidate (DMA), were purchased from Pierce Chemical Co.; fresh stock solutions were prepared immediately before use.

Cross-Linking. The cross-linking with DTBP was performed as described by Wang & Richards (1974). A solution of DTBP, adjusted to pH 8, was added to RCs (10 μM) containing cytochrome *c* (100 μM) in 10 mM Hepes, pH 8.0, and 0.025% LDAO to a final concentration of 2.0 mg/mL. The mixture was incubated for 1 h at T = 20 °C and then quenched by adding ammonium acetate (50 mM) to react with excess cross-linker and solid *N*-ethylmaleimide (5 mM) to prevent disulfide interchange. After 30 min of quenching, the sample was dialyzed at 4 °C overnight against 10 mM Tris-HCl, pH 8, and 0.025% LDAO. Cross-linkings with DMS and DMA were done similarly except that *N*-ethylmaleimide was omitted.

Cytochrome nitrophenyl azide, a gift of Drs. R. V. Lewis and W. S. Allison (UCSD), was obtained by reacting ethyl [(5-azido-2-nitrobenzoyl)amino]acetimidate (Az) with cyt *c* (horse) (Lewis & Allison, 1978). The derivatized cytochrome contained an average of 3.9 derivatized lysines per cytochrome (Lewis & Allison, 1978). Cross-linking between cytochrome and the RC was accomplished by illumination for 10 min with a Xenon arc (Bausch and Lomb Model 33-86-20-01, 100 W) blocked by a Corning 7-54 filter and heat-absorbing glass.

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis. Samples were heated in 1% $NaDodSO_4$ (65 °C, 60 min) and analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis using a 10% acrylamide gel, as described by Okamura et al. (1974). The gels were scanned either before or after being stained with Coomassie Brilliant Blue R-200 (Wilson Diagnostics) in a locally designed scanner on a Cary 14 optical spectrophotometer. This allowed precise quantitation of each band on the gel. Two-dimensional gel electrophoresis was performed according to the procedure of Wang & Richards (1974). The first dimension was run on a 0.75-mm slab (~11 × 4 cm) of 7.5% or 10% acrylamide (prepared with the same cross-linker composition as for the cylindrical gels, above), with a 2.5% acrylamide stacking gel. The slabs were run at 60 V until the tracking dye reached the desired position. A ~4-mm-wide strip was cut out from the slab with a scalpel and incubated in 1% DTT (Calbiochem) for 2 h. A second slab was prepared exactly as the first, except 0.1% DTT was added to the stacker after polymerization to ensure that the intermolecular cross-linker was fully cleaved. The strip of the gel cut from the first gel was secured to the top of the second slab with 1% agarose + 0.1% $NaDodSO_4$. This (second dimension) gel was run for the same time and at the same voltage as the previous (first dimension) gel.

All gels were fixed in 50% methanol + 5% acetic acid, stained in 0.25% Coomassie Blue R-200 in the fix solution, and destained in 10% methanol + 10% acetic acid. The cylindrical gels were stored in 7% acetic acid, and the slab gels were dried (under vacuum) onto filter paper after being destained.

Purification of the Cross-Linked Complex. The reaction mixture, containing RC, cytochrome, and the RC–cytochrome complex, was first passed through a Sephadex G-150 column (2 × 50 cm) equilibrated with 0.025% LDAO (10 mM Tris-HCl, pH 8) to separate the RC from free cytochrome. The RC fraction (~70 nmol) in the same buffer was then passed onto a column (1 × 15 cm) of cyt *c* bound to Sepharose 4B (4 mg/mL) prepared according to the procedure of Porath et al. (1973). The cross-linked RC–cyt complex passed through the column while un-cross-linked RCs bound to the column. The column was regenerated by elution with buffer of high ionic strength (0.1 M NaCl, 10 mM Tris-HCl, pH 8, and 0.025% LDAO).

Kinetic Measurements. The kinetic measurements of the electron transfer between D^+ and cytochrome were made with a kinetic spectrometer (T. Marinetti, D. Rosen, M. Okamura, and G. Feher, unpublished experiments), using a dye laser (Phase-R 2100C, λ_{max} = 590 nm, 0.3-μs duration). The sample was routinely monitored at 865 nm. The measurement of the rate of electron transfer from cyt c_2 was monitored at 431 nm to avoid fluorescence artifacts which occur on the microsecond time scale. Care was taken to expose the sample to the measuring beam only briefly (0.1 s) before the flash in order to avoid artifacts due to bleaching. The transient optical changes were digitized on a Nicolet 1090 digital oscilloscope and analyzed on a Cromemco Z2 computer. The log of the absorbance change vs. time was plotted, and the amplitudes and exponential decay times were determined graphically.

Antibody Inhibition. Antibodies specific against the L, M, and H subunits were prepared by affinity chromatography (Valkirs & Feher, 1982). Fab fragments were made by papain digestion (Press et al., 1966) to prevent the precipitation of RCs by antibodies. Purified IgG (0.7 mg/mL) was digested with papain (0.014 mg/mL) for 20 min at 37 °C in the presence of 10 mM cysteine, 2 mM ethylenediaminetetraacetic acid, and 50 mM phosphate buffer, pH 7.0. The reaction mixture was quenched with 50 mM iodoacetamide and dialyzed vs. 10 mM Tris-HCl, pH 8.0. RCs (0.1 μM) were incubated with different concentrations of Fab in a solution containing 100 mM NaCl, 10 mM reduced cytochrome (either *c* or c_2), 10 mM Tris-HCl, pH 8, and 100 μM Q-0 (2,3-dimethoxy-5-methyl-1,4-benzoquinone). The rate of reaction between RC and cytochrome was monitored after a laser flash (see above). In the absence of antibody, more than 95% of the decay of D^+ proceeded with a single exponential. Inhibition was indicated by the appearance of a slow phase in the rate of reaction between the RC and cytochrome. The antibody concentration was estimated by using an extinction coefficient of $\epsilon_{278} = 2.2 \times 10^5$ M^{-1} cm^{-1} (Little & Eisen, 1968).

Results

Cross-Linking of Reaction Centers with Cytochrome c and Cytochrome c_2 Using DTBP. The cleavable cross-linking agent dithiobis(propionimidate) [DTBP, introduced by Wang & Richards (1974)] was used for the majority of cross-linking experiments. This reagent cross-links protein by reacting with lysine residues. Reaction of DTBP with RCs alone resulted in the appearance of a major band labeled Y with an apparent molecular weight of 42 000 (see Figure 1b). The appearance of this new band was accompanied by the loss of L and H subunits (compare panels a and b of Figure 1) and was, therefore, identified as an LH dimer. This assignment was also confirmed by two-dimensional electrophoresis (see below). In addition, higher molecular weight bands were formed in lower and variable yields. Reaction of DTBP with cyt *c* (horse heart) alone resulted in the appearance of a band with an apparent molecular weight of 18 000 that is probably due to a cyt *c* dimer (data not shown).

When RCs were treated with DTBP in the presence of cyt *c*, two major cross-linked products appeared (see Figure 1c) in addition to the bands observed with RC or cyt *c* alone (see

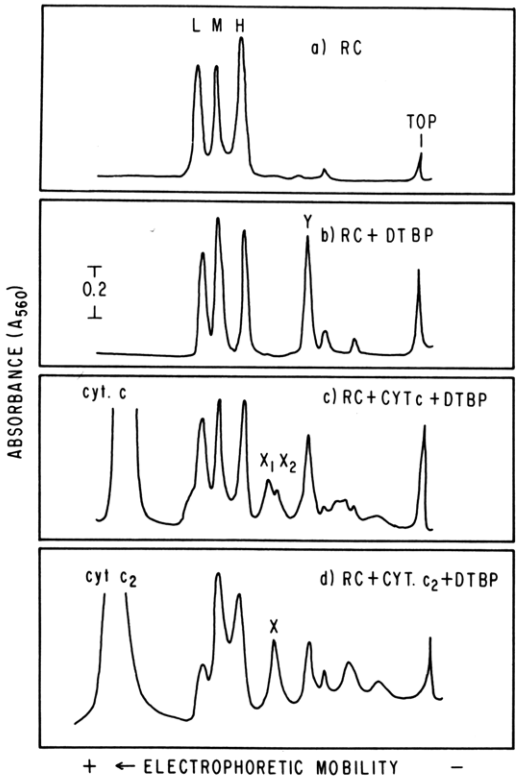

FIGURE 1: $NaDodSO_4$–polyacrylamide electrophoretogram of (a) the reaction center, (b) the reaction center cross-linked with DTBP, (c) the reaction center and cyt *c* (10 × excess) cross-linked with DTBP, and (d) the reaction center and cyt c_2 (10 × excess) cross-linked with DTBP (for details, see Experimental Procedures).

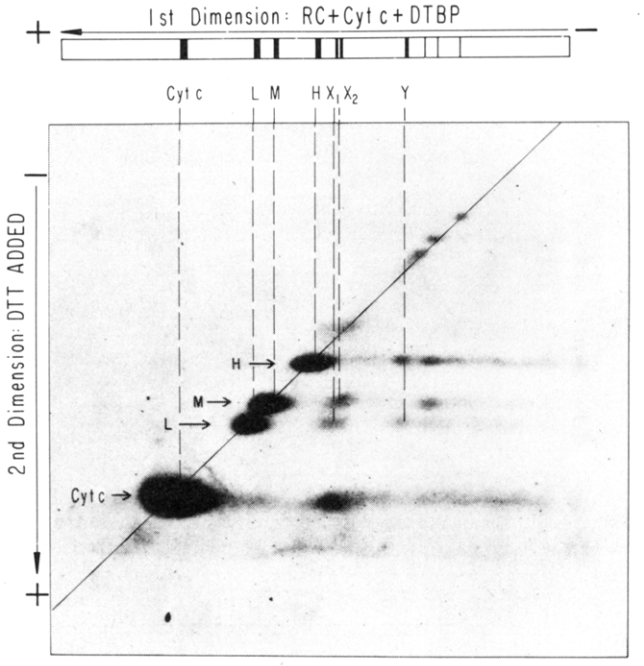

FIGURE 2: Two-dimensional $NaDodSO_4$–polyacrylamide gel electrophoresis analysis of the reaction center and cyt *c* cross-linked with DTBP. The first dimension corresponds to Figure 1c. The second dimension was run after the cross-linker was cleaved with DTT. The bands X_1 and X_2 cleave to produce L and M subunits plus cyt *c*. Cleavage of the high molecular weight products is also observed. The amount and distribution of the high molecular weight material varied from sample to sample (for details, see Experimental Procedures).

Figure 1b). These new bands had apparent molecular weights of 33 000 and 35 000 and were labeled X_1 and X_2, respectively. The amounts of X_1 and X_2 formed varied with different reaction conditions (e.g., DTBP and cytochrome concentrations), but the ratio of X_1 to X_2 remained approximately constant. The small amounts of product with higher molecular weights that were observed are probably due to higher aggregates of cross-linked subunits. Treatment of the cross-linked products with DTT resulted in the cleavage of the cross-linker.

When RCs were treated with DTBP in the presence of cyt c_2, a single new band (X) was observed; it had a mobility close to that of the bands previously observed. The amplitude of the L band was significantly decreased, suggesting that the X band was cyt c_2 cross-linked to the L subunit.

Two-Dimensional Gel Electrophoresis. The identity of the cytochrome-containing bands was determined by two-dimensional gel electrophoresis. After the reaction products were run in one dimension, the gel was treated with DTT to cleave the cross-linked products and run in the second dimension. The X_1 and X_2 bands split into L + cytochrome and M + cytochrome, respectively (see Figure 2). The gel patterns shown in Figure 2 were obtained after RCs were cross-linked with a 10-fold molar excess of cyt *c*. When equimolar amounts of cyt *c* and the RC (11 μM) were used, a similar pattern showing approximately equal cross-linking to L and M was obtained although the amount of cross-linking product was smaller.

The Y band cleaved to give L + H subunits, confirming the previous assignment. In addition, a higher molecular weight cross-linked product cleaved to give M + H subunits. In other gels, there appeared to be evidence for the formation of an LMH complex in this high molecular weight region also.

Two-dimensional gels of the RCs cross-linked to cyt c_2 with DTBP indicated that the X band was due to L–DTBP–cyt c_2 (data not shown) in accord with the assignment based on the decreased amplitude of L in the one-dimensional gel (Figure 1d).

Cross-Linking of RC with Cytochrome c Using Other Reagents. The cross-linking of RCs to horse heart cyt *c* was performed with several other cross-linkers. Two noncleavable imidate derivatives [dimethyl suberimidate (DMS) and dimethyl adipimidate (DMA)] that react with lysines and contain a bridging chain six and four carbons in length, respectively, were used. In addition, photoactivated aryl azide ethyl [(5-azido-2-nitrobenzoyl)amino]acetimidate (Az) was used. This reagent contains an imidate group which was first reacted with the lysines of cyt *c* (3.9 Az/cyt) (Lewis & Allison, 1978). The derivatized cytochrome was then illuminated in the presence of RCs, producing reactive nitrene intermediates that react rapidly ($<10^{-4}$ s) with nearby amino acid residues.

The results of the cross-linking reactions are shown in Figure 3. All of the cross-linkers produced two bands in the X region. DMS, which is the noncleavable analogue of DTBP, gave X_1 and X_2 bands of approximately equal intensities. DMA, which is two carbons shorter, produced a somewhat larger X_1 band, indicating that the lysines on L are nearer to the cytochrome binding site than those on M. The appearance of the X_1 and X_2 bands using the aryl azide cross-linker is particularly interesting because it does not require the presence of a nearby lysine residue; it reacts nonspecifically with amino acid residues on the RC.

Isolation and Photochemical Activity of the Cross-Linked Reaction Center–Cytochrome Complex. The cross-linked product was purified by gel filtration followed by affinity chromatography on a column containing cyt *c*. Unreacted RCs bound to the column (Figure 4a) while those RCs having cytochrome cross-linked at the active site passed through unretarded (Figure 4b).

Affinity-purified RC–cyt cross-linked with either DMS or DTBP contained ~1.0 cyt/RC (see Table I). The NaDod-

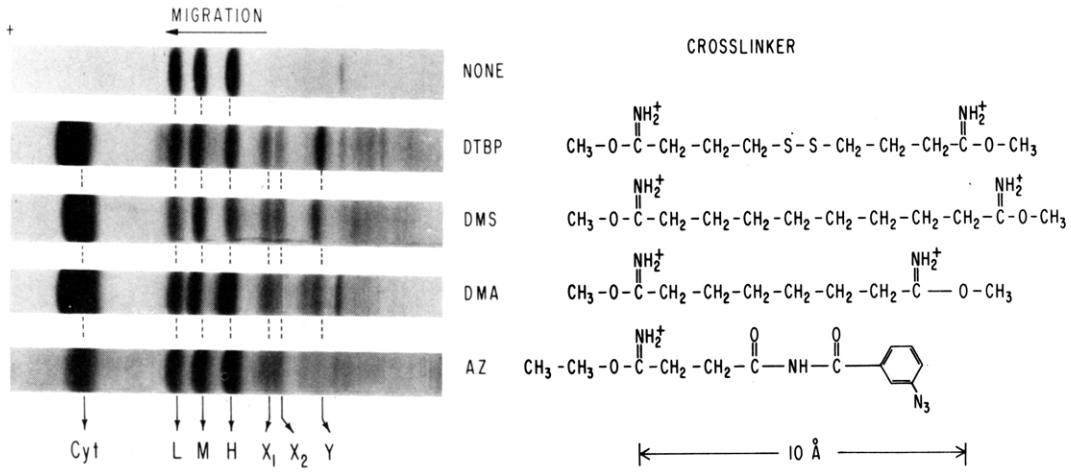

FIGURE 3: $NaDodSO_4$–polyacrylamide gel electrophoresis of reaction centers cross-linked to cyt *c* with various cross-linkers. The length of the cross-linker in the extended configuration was estimated from molecular models. Two bands in the X_1 and X_2 positions were observed in all cases although their relative amplitudes were different.

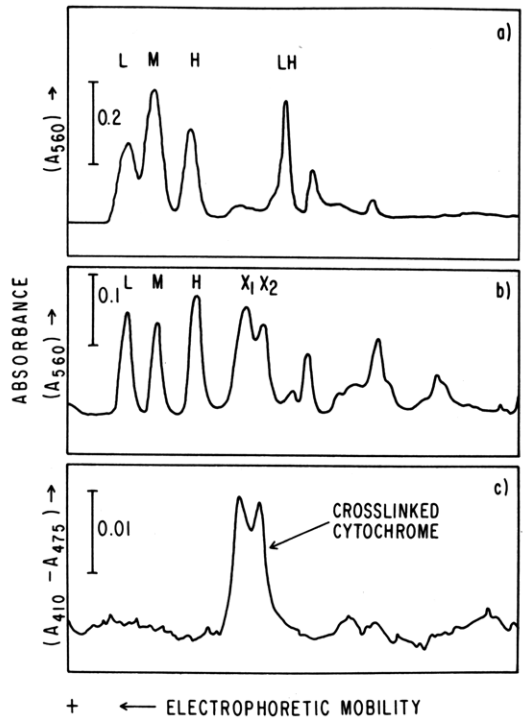

FIGURE 4: $NaDodSO_4$–polyacrylamide gel electrophoresis of fractions obtained in the purification process of the reaction center–DMS–cyt *c* complex. (a) Electrophoretogram of the fraction that bound to the cyt *c* chromatography column. The band marked LH corresponds to the Y band of Figure 1b. (b) Electrophoretogram of the fraction that did not bind to the cyt *c* chromatography column. Note the enhanced bands in the X_1 and X_2 positions (compare to unpurified mixture of Figure 1c). (c) Electrophoretogram of the same fraction as in (b) scanned at 410 and 475 nm. The difference in absorption at these two wavelengths is due to cyt *c*. Gels in (a) and (b) were stained with Coomassie Blue; the gel in (c) was not stained.

SO_4 gel patterns of the purified products cross-linked with DMS showed enhanced X_1 and X_2 bands (compare Figure 4b with Figure 1c). The DTBP gels (not shown) were similar but had more high molecular weight material due to higher aggregates that presumably resulted from disulfide reactions. The presence of bound cytochrome in the positions of the X bands was confirmed by observing the absorbance at 410 nm, the peak of the cytochrome Soret band (see Figure 4c).

The activities of the cross-linked products were assayed by optically monitoring at 865 nm the reduction of the oxidized primary donor subsequent to a short laser flash (Figure 5) (see Experimental Procedures). In the purified complex, the cyt *c* is oxidized, and consequently the rate of reduction of D^+ was

Table I: Kinetic Parameters[a] and Cytochrome Content of Cross-Linked Reaction Center–Cytochrome Complexes

cytochrome	cross-linker	τ_i	A_i (%)	cyt/RC[b]
cyt *c* (horse heart)	DTBP	50 μs	20	0.9
		320 μs	60	
		>10 ms	20	
	DMS	60 μs	25	1.0
		350 μs	60	
		>10 ms	15	
	none	20 μs	*c*	*c*
cyt c_2 (*R. sphaeroides*)	DTBP	1.2 μs	75	1.25
		>10 ms	25	
	DMS	1.0 μs	60	0.70
		>10 ms	40	
	none	1.2 μs	*c*	*c*

[a] The decay of D^+ was resolved into several exponentials of the form $A_i e^{-t/\tau_i}$. [b] Determined by the pyridine hemochromogen assay. [c] The amount of RC-cytochrome complex in the absence of cross-linker depends on the cytochrome concentration. Under the conditions of this experiment ([cyt] = 10 μM; [RC] = 3 μM), about 80–90% of the RCs contained a bound cytochrome.

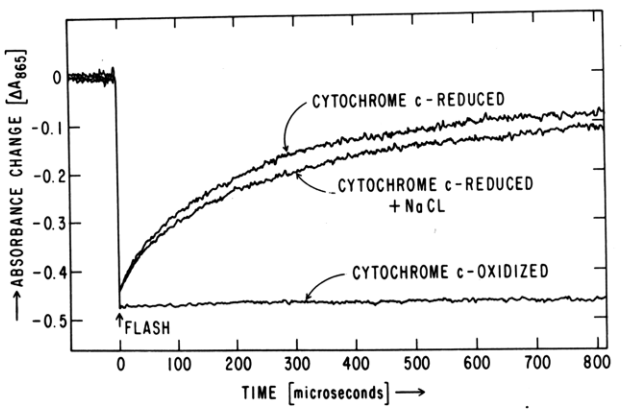

FIGURE 5: Flash-induced absorbance changes in the purified reaction center–DMS–cyt *c* complex. The purified sample (4 μM cross-linked reaction center in 10 mM Tris-HCl, pH 8, and 0.025% LDAO) contains oxidized cytochrome and shows a slow decay of D^+ due to electron transfer from A^-. Reduction of the cytochrome with 1 mM ascorbate produces a rapid decay of D^+ which remains essentially unchanged when 0.25 M NaCl is added. This rapid decay is indicative of electron transfer from the bound cytochrome.

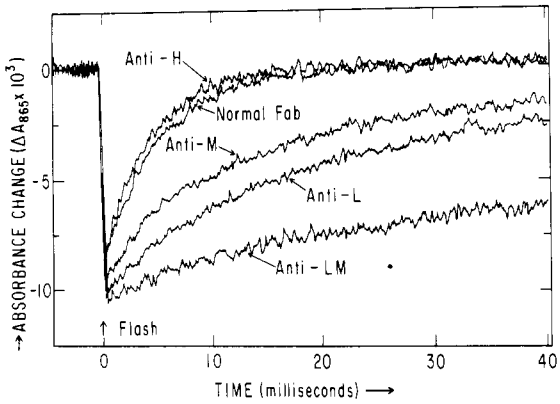

FIGURE 6: Kinetic assay of inhibition of electron transfer between reduced cyt c and D^+ following a laser flash. Reaction centers (0.11 μM) were incubated with Fab (~4 μM) in the presence of cyt c (10 μM) (100 mM NaCl, 10 mM Tris-HCl, pH 8, 0.025% LDAO, and 100 μM Q-0). The decay of the absorbance change at 865 nm due to the reaction between reduced cyt c and D^+ was slowed down by addition of Fab molecules that bind to L, M, or LM, but not by those that bind to H. Normal Fab was added as a control. Each trace is an average of four flashes (T = 20 °C).

found to be slow. Biphasic kinetics with characteristic times of ~100 ms and ~1.2 s, typical of back electron transfers from the primary and secondary quinones to D^+, were observed. Addition of ascorbate reduced the cytochrome with a concomitant increase in the rate of reduction of D^+. The kinetics of reduction of D^+ were resolved into several components. About 85% of the cross-linked cyt c was active, i.e., capable of reducing the oxidized donor (τ < 10 ms). The major component of the fast reaction had a characteristic time of 300 μs. A smaller amount of a faster component (~50 μs) was also observed. When the reaction was followed by monitoring the oxidation rate of cyt c at 550 nm (Parson, 1968), similar kinetics were observed, showing that the fast rate was due to electron transfer from cyt c. The addition of 0.25 M NaCl had little effect on the reaction rate between the cross-linked cytochrome and the reaction center (see Figure 5). For comparison, the reaction between excess cyt c and reaction centers in the un-cross-linked complex (Ke et al., 1970) occurred with τ = 20 μs at low salt concentrations. This time was found to be very dependent on ionic strength and cytochrome concentration. For instance, under the conditions of our assay (0.25 M NaCl, 5 μM cyt c), the electron transfer time for the un-cross-linked sample was found to be ~70 ms, i.e., much longer than in the cross-linked complex.

The cross-linked complex between RC and cyt c_2 was also isolated by affinity chromatography (see Table I) and was shown to be ~60–75% active. The electron transfer time for the reaction between cross-linked cyt c_2 and the RC was determined to be 1.2 ± 0.2 μs. Addition of 0.25 M NaCl had little effect on the rate of reaction in the cross-linked sample. The reaction time between RCs and noncovalently bound cyt c_2 at low ionic strength was found to be 1.0 ± 0.2 μs. This time increased to 5 ms in 0.25 M NaCl (5 μM cyt c_2).

Immunochemical Studies. In an alternate approach to determine which subunits are involved in the cytochrome binding, Fab fragments from antibodies directed against the individual subunits (L, M, H, and LM) were tested for their ability to block the electron transfer reaction between D^+ and cytochrome. Since only small amounts of affinity-purified antibodies were available, low concentrations (~0.1 μM) of reaction centers were used. In order to obtain an adequate signal-to-noise ratio of the optical transients at these low concentrations, the kinetics were slowed down by increasing the ionic strength (see previous section).

Table II: Antibody Inhibition of the Electron Transfer Reaction between D^+ and Reduced cyt c and cyt c_2[a]

antibody Fab[a]	cyt c		cyt c_2	
	A_s (% slow)	τ_s (ms)	A_s (% slow)	τ_s (ms)
anti-M_{153}	30	27	16	24
anti-M_{874}	25	10	18	12
anti-L_{870}	55	27	47	25
anti-L_{874}	17	21	25	19
anti-LM_{152}	25	120	19	110
anti-H_{793}	<5[b]	20	<5	20
normal	<5[b]	20	<5	20

[a] RCs (0.17 μM) were incubated with 1.6 μM Fab (~10-fold excess) in the presence of 10 μM cytochrome or 5 μM cytochrome c_2 (0.1 M NaCl, 10 mM Tris, pH 8, 0.025% LDAO, and 100 μM Q-0). Under these conditions, the reaction between D^+ and reduced cytochrome in the absence of antibodies took 4 and 2 ms for cytochrome c and cytochrome c_2, respectively. Inhibition was indicated by the appearance of biphasic kinetics with amplitude $A = A_f e^{-t/\tau_f} + A_s e^{-t/\tau_s}$. The amplitudes and times were estimated graphically from plots of log ΔA_{865} vs. time. The precision of A_s and τ_s was about 10% (except when A_s was given as a limit). [b] Limited by signal to noise ratio.

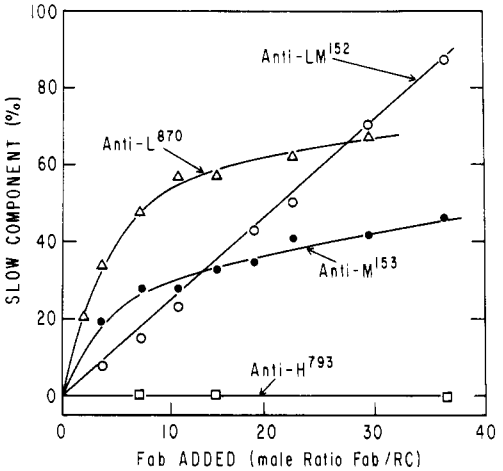

FIGURE 7: Percent slow phase of the kinetics of reduction of D^+ vs. amount of specific Fab added (obtained from experiments like those shown in Figure 6).

The results of the kinetics of D^+ reduction following a laser flash are shown in Figure 6. Inhibition by antibodies resulted in the appearance of a slow phase in the kinetics. The amplitude and decay time of the slow phase were obtained from plots of log absorbance vs. time (see Table II). The slow phase of the reaction in the presence of anti-LM had a characteristic time (τ ~ 110 ms) equal to that of the back-reaction between D^+ and Q_I^-, showing that cyt c was completely ineffective in reducing D^+. In the presence of anti-L and anti-M, the time was shorter (τ ~ 20 ms), indicating that the reaction between cytochrome and D^+ was strongly but not completely inhibited. In the presence of anti-H, there was no discernible slow phase, showing that no inhibition of electron transfer had occurred. A similar pattern of inhibition was observed for reaction with cyt c_2 (see Table II). A total of 11 anti-L, 9 anti-M, and 6 anti-H Fab preparations were tested. In all cases, anti-L and anti-M Fab inhibited the reaction while anti-H was not effective.

The inhibition was studied by using various concentrations of Fab (see Figure 7). The plot of percent inhibition (slow phase) vs. amount of Fab added shows that only a fraction of the total antibodies was directed to a cytochrome binding site. For instance, for a mole fraction of Fab/RC = 10, anti-LM^{152} Fab exhibited an ~30% slow component (see

Figure 7), indicating that only ~3% of these antibodies reacted specifically with the cytochrome binding site.

Discussion and Conclusion

In this study, chemical cross-linking and immunochemical techniques were used to localize the binding site for cytochrome on the bacterial reaction center. When cross-linking agents were used, horse heart cyt *c* was found to cross-link to the L and the M subunits, while cyt c_2 was found to cross-link mainly to the L subunit. In no case was cross-linking to the H subunit found despite the fact that H has more lysines than either L or M.

The simplest explanation of the results of the cross-linking experiment is that the cytochrome binding site is near (within ~10 Å) to both the L and the M subunits. The difference between the cross-linking of cyt *c* and cyt c_2 can be explained by differences in the number of lysines in the two proteins. Cytochrome *c* is a very basic protein with 19 lysine residues (Margoliash et al., 1961), whereas cyt c_2 has only 13 lysine groups (Ambler et al., 1979). Thus, cyt c_2 may lack lysine residues in the appropriate position to cross-link to the M subunit. It is, therefore, possible that cyt c_2 also binds to the M subunit but does not cross-link to it. Indeed, the antibody inhibition experiments to be discussed later indicate that this is the case. Another factor that could account for the additional cross-linking of horse heart cyt *c* is that the fit between this nonphysiological donor and the reaction center may not be as good as between cyt c_2 and the reaction center. This may impart a certain amount of motional freedom to cyt *c* in the binding site, thereby making additional cross-linkings available. This hypothesis is consistent with the kinetics of electron transfer in the cross-linked RC–cyt *c* complex (see below).

An alternative explanation for the differences in cross-linking is that cyt *c* and c_2 bind at different sites. This, however, is incompatible with the results of previous equilibrium dialysis studies which have shown that both cyt *c* and c_2 bind at the same site (Rosen et al., 1980).

The electron transfer rates of the cross-linked cytochromes give some indication about the binding of the cyt in the active site. For horse cyt *c*, the rate of reaction in the majority of the cross-linked species is slower by a factor of 15 (τ = 300 μs) than the rate observed in the un-cross-linked RC–cyt *c* complex (τ = 20 μs). This reduction in rate indicates that cyt *c* is cross-linked in a position that is not optimal for electron transfer. Studies of the electron transfer kinetics showed that the interaction between cyt *c* and D^+ is strongly viscosity dependent (T. D. Marinetti, D. Rosen, M. Y. Okamura, and G. Feher, unpublished experiments). This suggests that an electrostatic interaction results in a complex in which rotational motion may be required to achieve the proper orientation for electron transfer.

In contrast, the cross-linked cyt c_2 exhibits almost the identical rate (τ = 1.1 μs) of electron transfer as the native complex (τ = 1.0 μs), indicating that there is good matching in the cross-linked RC–cyt c_2 complex. This is in accord with the results of kinetic studies which showed that the interaction between cyt c_2 and RCs is viscosity independent (T. D. Marinetti, D. Rosen, M. Y. Okamura, and G. Feher, unpublished experiments), suggesting that a tight, well-defined complex between RC and cyt c_2 is formed.

Antibody inhibition of the electron transfer reaction between D^+ and cytochrome supports the findings from the cross-linking experiments. Fab fractions of anti-L, anti-M, and anti-LM block the reaction of both cyt *c* and cyt c_2 with D^+. Anti-H had no effect, despite the fact that it binds to RC very effectively (Valkirs & Feher, 1982). These results indicate that the cyt binding site for both cyt *c* and cyt c_2 is near both the L and M subunits, but far from the antigenic sites of the H subunit. Since the binding surface of the Fab unit has a cross-sectional diameter of at least 35 Å (Dickerson & Geis, 1969), the resolution of this technique is lower than in the cross-linking experiment. The kinetics of the reaction between cyt *c* and D^+ in the presence of anti-L and anti-M ($\tau \simeq 20$ ms) suggest that the accessibility of cyt *c* is reduced by these antibodies but not completely eliminated as is the case with anti-LM (τ = 110 ms). Anti-LM probably binds closer to the cytochrome binding site, possibly to antigenic determinants from both L and M subunits.

Information about the topology of the RC in the membrane can be inferred from the cross-linking studies. Prince et al. (1975) have shown that the cytochrome binding site is located at the interior surface of the chromatophore membrane (periplasmic side). The cross-linking of cyt *c* to L and M subunits leads to the conclusion that L and M are exposed at the membrane surface on the interior of the chromatophore. This conclusion is also in agreement with other labeling experiments [see, for example, Valkirs & Feher (1982)].

The proximity of the bound cytochrome to both L and M suggests that the binding site is located in a cleft between the two subunits. However, in view of the limited resolution (~10 Å) of our experiments, the actual contact between the reaction center and cytochrome may occur at only one of the two subunits. A similar "cleft" model, based on chemical cross-linking studies, has been suggested for the binding site for cyt *c* to cytochrome oxidase [e.g., see Capaldi et al. (1982)]. Since the reaction center and cytochrome oxidase serve analogous functions in electron transport, it would not be surprising if they showed structural similarities.

Added in Proof

The proximity of bound cyt c_2 to the L and M subunits is supported by studies in which the carboxyl groups on the RC were modified with the reagent 1-ethyl-3-[3-(trimethylamino)propyl]carbodiimide (Okamura & Feher, 1983).

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References

Ambler, R. P., Daniel, M., Hermoso, J., Meyer, T. E., Bartsch, R. G., & Kamen, M. D. (1979) *Nature (London) 278*, 659–660.

Bartsch, R. (1963) in *Bacterial Photosynthesis* (Gest, H., San Pietro, A., & Vernon, L. P., Eds.) Antioch Press, Yellow Springs, OH.

Bartsch, R. (1978) in *The Photosynthetic Bacteria* (Clayton, R. K., & Sistrom, W. R., Eds.) pp 249–279, Plenum Press, New York.

Capaldi, R. A., Darley-Usmar, V., Fuller, S., & Millet, F. (1982) *FEBS Lett. 138*, 1–7.

Das, M., & Fox, C. F. (1979) *Annu. Rev. Biophys. Bioeng. 8*, 165–193.

Dickerson, K. E., & Geis, I. (1969) *The Structure and Action of Proteins*, Harper and Row, New York.

Dutton, P. L., & Prince, R. C. (1978) in *The Photosynthetic Bacteria* (Clayton, R. K., & Sistrom, W. R., Eds.) pp

525-570, Plenum Press, New York.
Feher, G., & Okamura, M. Y. (1978) in *The Photosynthetic Bacteria* (Clayton, R. K., & Sistrom, W. R., Eds.) pp 349-386, Plenum Press, New York.
Ke, B., Chaney, T. H., & Reed, D. W. (1970) *Biochim. Biophys. Acta 216*, 373-383.
Lewis, R. V., & Allison, W. S. (1978) *Arch. Biochem. Biophys. 190*, 163-173.
Little, J. R., & Eisen, N. H. (1968) *Biochemistry 7*, 711-720.
Margoliash, E., & Frohwirt, N. (1959) *Biochem. J. 71*, 570-572.
Margoliash, E., Smith, E. L., Kreil, G., & Tuppy, H. (1961) *Nature (London) 192*, 1125-1127.
Marinetti, T. D., Okamura, M. Y., & Feher, G. (1979) *Biochemistry 18*, 3126-3133.
Okamura, M. Y., & Feher, G. (1983) *Biophys. J.* (Abstr.) (in press).
Okamura, M. Y., Steiner, L. A., & Feher, G. (1974) *Biochemistry 13*, 1394-1403.
Overfield, R. E., Wraight, C. A., & Devault, D. (1979) *FEBS Lett. 105*, 137-142.
Parson, W. W. (1968) *Biochim. Biophys. Acta 153*, 248-259.
Porath, J., Aspberg, K., Dreven, H., & Axen, R. (1973) *J. Chromatogr. 86*, 53-56.
Press, E. M., Piggot, P. J., & Porter, R. R. (1966) *Biochem. J. 99*, 356.
Prince, R. C., Baccarini-Melandri, A., Hauska, G. A., Melandri, B. A., & Crofts, A. R. (1975) *Biochim. Biophys. Acta 387*, 212-227.
Rosen, D., Okamura, M. Y., & Feher, G. (1979) *Biophys. J. 25*, 204a.
Rosen, D., Okamura, M. Y., & Feher, G. (1980) *Biochemistry 19*, 5687-5692.
Straley, S. C., Parson, W. W., Mauzerall, D. C., & Clayton, R. K. (1973) *Biochim. Biophys. Acta 305*, 597-609.
Valkirs, G., & Feher, G. (1982) *J. Cell Biol. 95*, 179-188.
Wang, K., & Richards, F. M. (1974) *Isr. J. Chem. 249*, 8005-8018.

Purification and Characterization of a Scallop Sarcoplasmic Calcium-Binding Protein†

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ABSTRACT: A sarcoplasmic Ca^{2+}-binding protein (SCBP) from scallop striated muscle has been purified to homogeneity by chromatography on Sephacryl S-200 and diethylaminoethylcellulose. It appeared to be greater than 95% pure on sodium dodecyl sulfate gel electrophoresis, with a mobility corresponding to M_r ~22000. The amino terminus of SCBP appears to be blocked, as judged by automated sequencer analysis. Amino acid analysis indicates that SCBP is similar to other invertebrate sarcoplasmic Ca^{2+}-binding proteins. The amino acid compositions of SCBP, scallop calmodulin, and scallop troponin C are all quite different, showing that these three Ca^{2+}-binding proteins are distinct entities. The Ca^{2+}- and Mg^{2+}-binding properties of SCBP were investigated by measuring the decreases in tryptophan fluorescence upon titration with these cations. There appears to be two classes of Ca^{2+}-binding sites, with different affinities and specifications: one class binds either Ca^{2+} (with $K > 10^8$ M^{-1}) or Mg^{2+} (with $K \sim 4 \times 10^4$ M^{-1}), and the other class specifically binds Ca^{2+} (with $K \sim 3 \times 10^6$ M^{-1}). These values are very similar to those for the Ca^{2+}-Mg^{2+} and the Ca^{2+}-specific sites found in rabbit skeletal muscle troponin [Potter, J. D., & Gergely, J. (1975) *J. Biol. Chem. 250*, 4628-4633]. Circular dichroic studies show that SCBP contains about 35% α helix in the absence of Ca^{2+} and Mg^{2+}. The helical content can be increased to about 40% by adding Ca^{2+}. Mg^{2+} addition produces only about half of this increase in α helix, consistent with the observation that SCBP contains both Ca^{2+}-Mg^{2+} and Ca^{2+}-specific binding sites. Although the function of SCBP is unknown, the presence of Ca^{2+}-specific sites on this protein suggests that it may play a direct role in the regulation of scallop muscle contraction.

Muscle cells contain a homologous group of Ca^{2+}-binding proteins that includes troponin C, myosin light chains, and parvalbumin. Also related to these are calmodulin (which appears to be present in all cells), brain S-100 protein, and intestinal vitamin D dependent Ca^{2+}-binding proteins [for reviews, see Collins (1976), Barker et al. (1978), Kretsinger et al. (1980), and Wnuk et al. (1982)]. Although crystallizations of several of these proteins have been reported (Kretsinger et al., 1980; Mercola et al., 1975; Strasburg et al., 1980), to date, a parvalbumin from carp is the only member of this family to have its three-dimensional structure determined by X-ray diffraction studies (Kretsinger & Nockolds, 1973). The structure of the carp parvalbumin has formed the basis for many predictions and speculations regarding the structures of the related Ca^{2+}-binding proteins [e.g., see Collins et al. (1973), Weeds & McLachlan (1974), Kretsinger & Barry (1975), Reid & Hodges (1980), and Bagshaw & Kendrick-Jones (1980)].

Parvalbumins appear to be absent from invertebrate sarcoplasm, and instead a new class of muscle calcium-binding proteins has been found in crayfish (Cox et al., 1976; Wnuk et al., 1979, 1981), scallops (Lehman & Szent-Györgyi, 1975), the protochordate *Amphioxus* (Kohler et al., 1978), and

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