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# Photoinduced Electron Transfer within Complexes between Plastocyanin and Ruthenium Bisbipyridine Dicarboxybipyridine Cytochrome *c* Derivatives†

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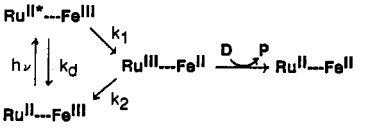
ABSTRACT: A new technique has been developed to measure intracomplex electron transfer between cytochrome *c* and its redox partners. Cytochrome *c* derivatives labeled at single lysine amino groups with ruthenium bisbipyridine dicarboxybipyridine were prepared as previously described [Pan, L. P., Durham, B., Wolinska, J., & Millett, F. (1988) *Biochemistry 27*, 7180-7184]. Excitation of $Ru^{II}$ with a short light pulse resulted in the formation of the excited-state $Ru^{II*}$, which rapidly transferred an electron to the ferric heme group to form $Fe^{II}$ and $Ru^{III}$. Aniline was included in the buffer to reduce $Ru^{III}$ to $Ru^{II}$, leaving the heme group in the ferrous state. This process was complete within the lifetime of the light pulse. When plastocyanin was present in the solution, electron transfer from the ferrous heme of cytochrome *c* to $Cu^{II}$ in plastocyanin was observed. All of the ruthenium cytochrome *c* derivatives formed electrostatic complexes with plastocyanin at low ionic strength, allowing intracomplex electron-transfer rate constants to be measured. The rate constants for derivatives modified at the indicated lysines were as follows: Lys 13, 1920 $s^{-1}$; Lys 8, 1480 $s^{-1}$; Lys 7, 1340 $s^{-1}$; Lys 86, 1020 $s^{-1}$; Lys 25, 820 $s^{-1}$; Lys 72, 800 $s^{-1}$; Lys 27, 530 $s^{-1}$. It is interesting that the derivative modified at lysine 13 at the top of the heme crevice had the largest rate constant, while lysine 27 at the right side of the heme crevice had the smallest. One possible explanation is that the bulky ruthenium complex at lysine 13 caused plastocyanin to bind toward the bottom of the heme crevice in a more favorable orientation for rapid electron transfer. The intracomplex rate constants for the derivatives modified at lysines 13 and 27 were actually larger at an ionic strength of 30 mM than at 5 mM, even though the binding constants were much smaller. High ionic strength may allow a greater range of binding orientations to occur, some of which are more favorable for rapid electron transfer.

Despite the importance of biological electron-transfer reactions, relatively few techniques are available to measure the rate of the actual electron-transfer step within a complex between two proteins. This is because the reactions are usually too rapid for stopped-flow techniques, and protein binding and dissociation steps are frequently rate-limiting. Several approaches have been developed to address this problem. Simondsen et al. (1982) developed the use of flavins to photochemically reduce the redox centers and initiate intracomplex electron transfer. This technique has been applied to a number of systems, including the complex between cytochrome *c* and cytochrome *c* peroxidase (Hazzard et al., 1987a,b, 1988a,b). Pulse radiolysis has been used to initiate electron transfer within the cytochrome *c*–cytochrome $b_5$ complex and the cytochrome *c*–plastocyanin complex (McLendon & Miller, 1985; Peerey & Kostič, 1989). In another approach, replacement of the native heme group with a zinc porphyrin allows electron transfer to be initiated photochemically. This technique was used to study electron transfer between cytochrome *c* and

Scheme I

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zinc–cytochrome *c* peroxidase and between zinc–cytochrome *c* and cytochrome $b_5$ (Liang et al., 1987; McLendon et al., 1985).

We have recently introduced a new class of cytochrome *c* derivatives that are labeled at single lysine amino groups with ruthenium bisbipyridine dicarboxybipyridine [$Ru^{II}(bpy)_2$-(dcbpy)[1] (Pan et al., 1988)]. Ten singly labeled cytochrome *c* derivatives have now been purified and characterized (Durham et al., 1989). The $Ru^{II}$ group can be photoexcited to a metal-to-ligand charge-transfer state, $Ru^{II}$*, which is a strong reducing agent and can transfer an electron to the heme group $Fe^{III}$ of cytochrome *c* (Scheme I). Since $Ru^{III}$ is a powerful oxidizing agent, a rapid back electron transfer reaction normally occurs, producing $Ru^{II}$ and $Fe^{III}$. The rate constants were found to be $k_1 = 14 \times 10^6$ s$^{-1}$ and $k_2 = 24 \times 10^6$ s$^{-1}$ for the derivative modified at lysine 72, which has a distance of 8–16 Å between the ruthenium and heme groups (Durham et al., 1989). The $k_1$ rate constants ranged from $20 \times 10^6$ s$^{-1}$ for the derivative modified at lysine 13, 6–10 Å from the heme crevice, down to $10^5$ and less for the derivatives modified at lysines farther from the heme group.

In the present paper, we describe a new technique to measure electron transfer in 1:1 complexes between $Ru(bpy)_2$-(dcbpy)–cytochrome *c* derivatives and plastocyanin (Scheme I). A short light pulse was used to excite $Ru^{II}$ to $Ru^{II}$*, which then rapidly transferred an electron to the heme $Fe^{III}$. Aniline was included in the buffer to act as a sacrificial electron donor, D, to reduce $Ru^{III}$ to $Ru^{II}$ and form oxidation products P. This prevented the back reaction represented by $k_2$ in Scheme I. The heme $Fe^{II}$ then transferred an electron to $Cu^{II}$ in plastocyanin. All of the $Ru(bpy)_2$(dcbpy)–cytochrome *c* derivatives formed 1:1 complexes with plastocyanin at low ionic strength that allowed intracomplex electron-transfer rates to be measured. This new technique appears to be generally applicable to the electron-transfer reactions of cytochrome *c*.

## Experimental Procedures

*Materials.* Horse heart cytochrome *c* was obtained from Sigma Chemical Co. (type VI). The $Ru(bpy)_2$(dcbpy)–cytochrome *c* derivatives were prepared as described by Pan et al. (1988) and Durham et al. (1989). Plastocyanin was prepared from spinach leaves by the method of Geren et al. (1983).

*Transient Absorption Kinetics.* Transient absorbance measurements were carried out by flash photolysis of 300-μL solutions contained in a 1-cm glass semimicro cuvette. The excitation light flash was provided by a xenon flash lamp placed 2 cm from the cuvette and passed through a glass filter to remove light with wavelengths shorter than 330 nm. The pulse width of the flash lamp was variable, with associated circuitry designed to cut the lamp off rapidly at the end of the pulse. The probe beam came from a tungsten halogen lamp and was passed through a 500-nm high pass filter, collimated, and focused on the sample cuvette by a fused silica lens. The probe beam was limited to <1 s by an electronically controlled shutter synchronized with the xenon flash lamp. The probe beam passed through a monochromator (Kratos GM252) and was detected with an R 446 photomultipier tube. A DC offset was added to the signal through a summing amplifier of local design. The signal was recorded on a 1010 Biomation waveform recorder and transferred to an IBM PC for kinetic analysis. The reaction solutions typically contained 5 μM cytochrome *c* derivative, 5–40 μM plastocyanin, 10 mM aniline, and 20 nM cytochrome oxidase in 5 mM sodium phosphate, pH 7.0 at 25 °C. The reaction of cytochrome *c* was monitored at 550 nm. The reaction curve was fitted to a first-order decay curve with an apparent rate constant $k_{obs}$ by using a weighted linear least-squares program.

## Results

It was necessary in these experiments to use a sacrificial redox agent to reduce $Ru^{III}$ to $Ru^{II}$ and prevent the back reaction represented by $k_2$ in Scheme I. EDTA has been widely used as a sacrificial reductant in studying the photochemistry of $Ru(bpy)_3$ and has been found to react with $Ru^{III}(bpy)_3$ to form $Ru^{II}(bpy)_3$ and oxidation products of EDTA (Miller & McLendon, 1981). The heme group $Fe^{III}$ of all the $Ru(bpy)_2$(dcbpy)–cytochrome *c* derivatives was reduced when subjected to a short light pulse in the presence of EDTA. However, a limitation to the use of EDTA was that the concentration necessary for efficient photoreduction, 10 mM, made a significant contribution to the ionic strength and interfered with the formation of a tight electrostatic complex with plastocyanin. Aniline was found to be more effective than EDTA in reducing $Ru^{III}$ to $Ru^{II}$ and promoting photoreduction of the heme group. Excitation of the lysine 13 cytochrome *c* derivative with a short light flash in the presence of aniline resulted in rapid reduction of the heme group iron (Figure 1A). Photoreduction was complete within the pulse width of the flash lamp, even for pulses as short as 1 μs. However, the extent of reduction increased with the pulse width of the light flash, as would be expected if a number of reduction–reoxidation cycles occurred before the heme was "trapped" in the reduced state (Scheme I). Therefore, the pulse width of the flash was adjusted so that it was short compared to the rate of the subsequent electron-transfer reaction but long enough to give a transient with a good signal-to-noise ratio. Previous studies have shown that photoexcitation of ruthenium does not lead to oxidation of nearby amino acid side chains and that the rate constants $k_1$ and $k_2$ were unaffected by repeated light excitation (Durham et al., 1989). The extent of photoreduction was smaller for derivatives with a greater separation between the ruthenium and heme groups, but this distance dependence was not nearly as large as it was for the rate constants $k_1$ and $k_2$ (Durham et al., 1989). No photoreduction of native cytochrome *c* was observed under these conditions, since the flash lamp was filtered to remove light with wavelengths shorter than 330 nm. Aniline did not quench the luminescence of the excited state of ruthenium, $Ru^{II}$*, or inhibit the reaction of the cytochrome *c* derivatives with plastocyanin or cytochrome oxidase. (Dimethylamino)benzoate was also found to be an effective sacrificial reducing agent for these derivatives.

Excitation of a solution containing the lysine 13 cytochrome *c* derivative and plastocyanin resulted in rapid photoreduction of the heme group in cytochrome *c* followed by electron transfer to the copper atom in plastocyanin (Figure 1B). No direct electron transfer from $Ru^{II}$* to the copper in plastocyanin was detected. The photolysis transient could be repeated hundreds of times in the presence of catalytic concentrations of cytochrome oxidase to reoxidize cytochrome *c* (and hence plastocyanin) between pulses. The pseudo-first-order rate constant, $k_{obs}$, for electron transfer to plastocyanin remained the same after repeated light flashes, indicating that there was no light-induced degradation of either the ruthenium cytochrome *c* derivative or plastocyanin. $k_{obs}$ was independent of the concentration of aniline and was the same when (dimethylamino)benzoate was substituted for aniline, indicating that the sacrificial reductant did not affect the reaction. The

[1] Abbreviations: bpy, 2,2′-bipyridine; dcbpy, 4,4′-dicarboxy-2,2′-bipyridine; CDNP, carboxydinitrophenyl; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide.

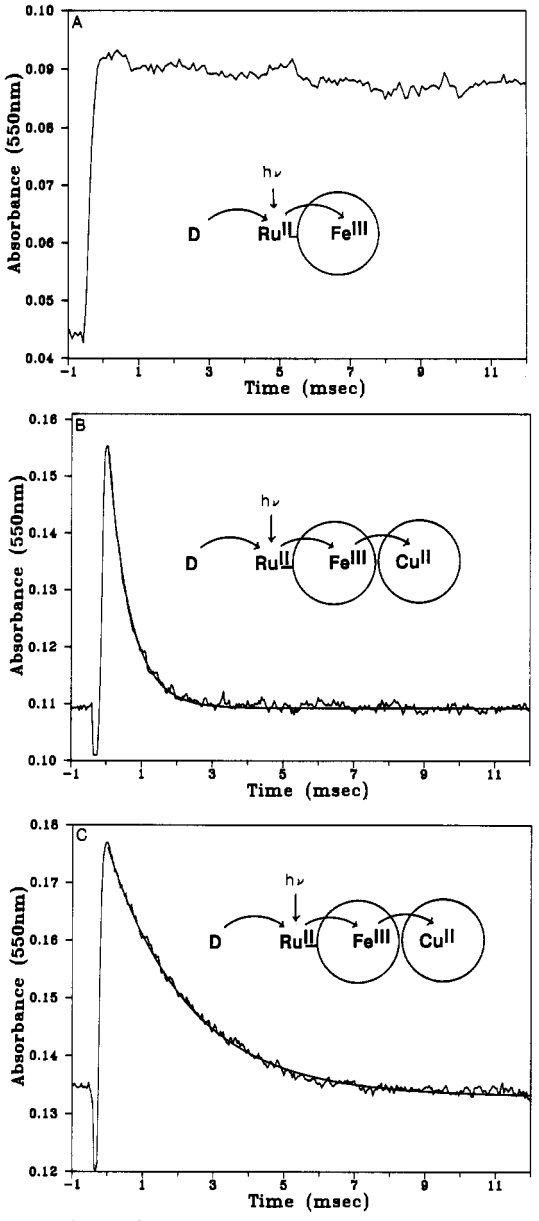
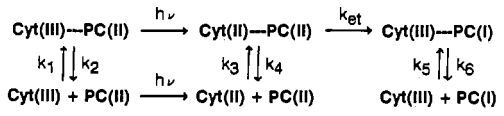



FIGURE 1: Photoinduced electron transfer from $Ru(bpy)_2(dcbpy)$–cytochrome $c$ derivatives to plastocyanin. All samples were in 2 mM sodium phosphate buffer, pH 7, containing 10 mM aniline and 20 nM cytochrome oxidase at 25 °C. The sample cuvette was subjected to a short light flash, and the redox state of the cytochrome $c$ heme group was followed at 550 nm. (A) Photoreduction of 5 $\mu$M lysine 13 derivative. (B) Oxidation of photoreduced lysine 13 derivative (5 $\mu$M) by 30 $\mu$M plastocyanin. The oxidation transient was fitted by a first-order decay with a $k_{obs}$ of 1600 $s^{-1}$. (C) Oxidation of photoreduced lysine 27 derivative by 50 $\mu$M plastocyanin. $k_{obs}$ was 400 $s^{-1}$.

Scheme II




dependence of $k_{obs}$ on the concentration of plastocyanin was found to be hyperbolic, suggesting that the reaction was mediated by a 1:1 complex between the two proteins (Figure 2). The rate constant data were fit to

$$k_{obs} = k_{et}K_A[P]/(1 + K_A[P]) \quad (1)$$

where $k_{et}$ is the rate of electron transfer between the heme group and the copper atom in a 1:1 complex, $K_A$ is the binding

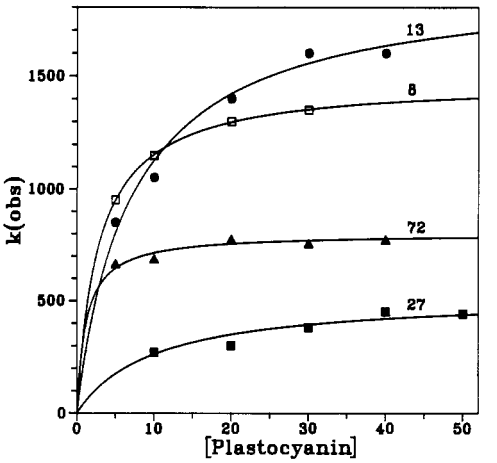



FIGURE 2: Kinetics of electron transfer from $Ru(bpy)_2(dcbpy)$–cytochrome $c$ derivatives to plastocyanin at low ionic strength. The pseudo-first-order rate constants for the oxidation of the heme $Fe^{II}$ in the cytochrome $c$ derivatives (5 $\mu$M) were measured under the conditions given in Figure 1 and plotted as a function of the concentration of plastocyanin. The solid lines are the best fits of eq 1 to the rate constants, with the kinetic parameters given in Table I. The rate constants are shown for derivatives modified at lysines 13 (●), 8 (□), 72 (▲), and 27 (■).

Table I: Rate Constants for Intracomplex Electron Transfer between $Ru(bpy)_2(dcbpy)$–Cytochrome $c$ Derivatives and Plastocyanin[a]

| lysine labeled | ionic strengh (mM) | $k_{et}$ ($s^{-1}$) | $K_A$ [$\times 10^{-4}$ ($M^{-1}$)] |
|---|---|---|---|
| 7 | 5 | 1340 ± 110 | 14 ± 4 |
| 8 | 5 | 1480 ± 120 | 33 ± 10 |
| 13 | 5 | 1920 ± 150 | 14 ± 4 |
| 13 | 32 | 4000 ± 1200 | 0.4 ± 0.2 |
| 25 | 5 | 820 ± 60 | 20 ± 5 |
| 27 | 5 | 530 ± 70 | 10 ± 2 |
| 27 | 56 | 2500 ± 1000 | 0.4 ± 0.2 |
| 72 | 5 | 800 ± 50 | 100 ± 50 |
| 72 | 11 | 730 ± 40 | 14 ± 3 |
| 72 | 110 | 700 ± 300 | 0.5 ± 0.2 |
| 86 | 5 | 1025 ± 100 | 25 ± 5 |

[a] The kinetic constants were measured by fitting eq 1 to $k_{obs}$ vs [PC] plots as shown in Figures 2 and 3.

constant of the complex, and [P] is the concentration of plastocyanin. This rate equation is based on the mechanism shown in Scheme II, with $K_A = k_3/k_4$ (Peerey & Kostić, 1989). The best fit of the data shown in Figure 2 was obtained with $k_{et} = 1920\ s^{-1}$ and $K_A = 1.4 \times 10^5\ M^{-1}$. The electron-transfer reaction of the lysine 8 derivative was nearly as fast of that of the lysine 13 derivative, while the reaction involving the lysine 27 derivative was significantly slower, with $k_{et} = 530\ s^{-1}$ and $K_a = 1 \times 10^5\ M^{-1}$ (Figures 1C and 2; Table I). The derivatives modified at lysines 7, 25, 72, and 86 had intracomplex electron-transfer rate constants ranging from 800 to 1340 $s^{-1}$ (Table I). Peerey and Kostić (1989) have found that the rate constant for native cytochrome $c$ under similar conditions was $k_{obs} = 1050\ s^{-1}$. Increasing the ionic strength decreased the binding constant $K_A$ of the lysine 72 derivative but did not affect $k_{et}$ (Figure 3; Table I). However, the electron-transfer rate constant for the lysine 27 derivative was increased to 2500 ± 1000 $s^{-1}$ at 40 mM ionic strength and high concentrations of plastocyanin (Figure 3; Table I). The concentration dependence of $k_{obs}$ was nearly second order, leading to relatively large errors in the estimated values of the kinetic constants. A similar result was observed for the lysine 13 derivative, where the reaction became nearly second order at 30 mM ionic strength, with a value for $k_{et}$ of 4000 ± 1200. The pseudo-first-order rate constants of all the derivatives

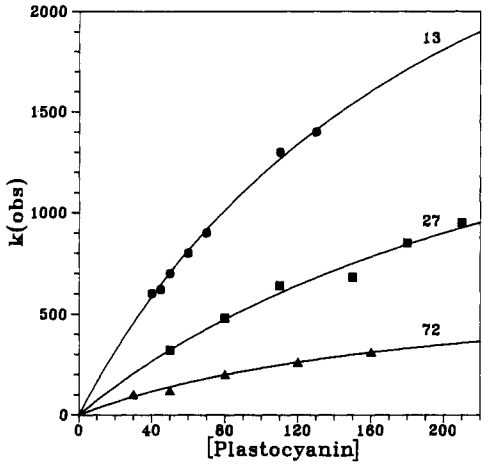



FIGURE 3: Kinetics of electron transfer from $Ru(bpy)_2(dcbpy)$–cytochrome *c* derivatives to plastocyanin at high ionic strength. Rates are shown for derivatives modified at lysine 13, $I$ = 32 mM (●), lysine 27, $I$ = 56 mM (■), and lysine 72, $I$ = 110 mM (▲). The conditions were the same as described in Figure 1 except for addition of sodium chloride to adjust the ionic strength $I$.

measured at a fixed concentration of plastocyanin (30 $\mu$M) were found to decrease with increasing ionic strength, as expected for a reaction facilitated by electrostatic interactions (Figure 4).

## Discussion

The singly labeled $Ru(bpy)_2(dcbpy)$–cytochrome *c* derivatives provide a different approach to the study of intracomplex electron transfer that offers several attractive features. First, the pathway for reduction of the heme $Fe^{III}$ by the photoexcited $Ru^{II*}$ is well-defined, and the rate is rapid compared to subsequent electron-transfer steps (Durham et al., 1989). Furthermore, the rate of reduction of the heme does not appear to be inhibited by complex formation with other proteins. Complex formation with cytochrome oxidase inhibited the rapid reduction of cytochrome *c* by pulse radiolysis (Veerman et al., 1982) preventing the measurement of intracomplex electron transfer by this technique. Photoinduced electron transfer between the $Ru(bpy)_2(dcbpy)$–cytochrome *c* derivatives and cytochrome oxidase has been detected in 1:1 complexes at low ionic strength (Pan et al., unpublished results). Second, the attached $Ru(bpy)_2(dcbpy)$ group is highly selective in the reduction of the heme group. No direct reduction of the copper in plastocyanin was observed for any of the derivatives. Third, the ruthenium electron-transfer chemistry lends itself to the use of a variety of sacrificial redox agents. Both aniline and (dimethylamino)benzoate were found to act as efficient electron donors in the present experiments, without affecting the rate of electron transfer from cytochrome *c* to plastocyanin. A detailed study of the reactions of sacrificial reductants and oxidants is currently underway. Fourth, the $Ru(bpy)_2(dcbpy)$–cytochrome *c* derivatives all have the same heme redox potentials and visible spectra as native cytochrome *c*, including the 695-nm absorbance band assigned to the bond between Met 80 and heme iron (Pan et al., 1988). The heme redox potential and the 695-nm absorbance band are both highly sensitive to protein conformational changes in the heme crevice region resulting from chemical modification or mutation (Pearce et al. 1989; Louie et al., 1988). The derivatives are thus likely to have nearly the same conformation in the heme crevice region as native cytochrome *c*. It is possible, of course, that ruthenium modification could have caused more localized changes in conformation; 500-MHz NMR studies

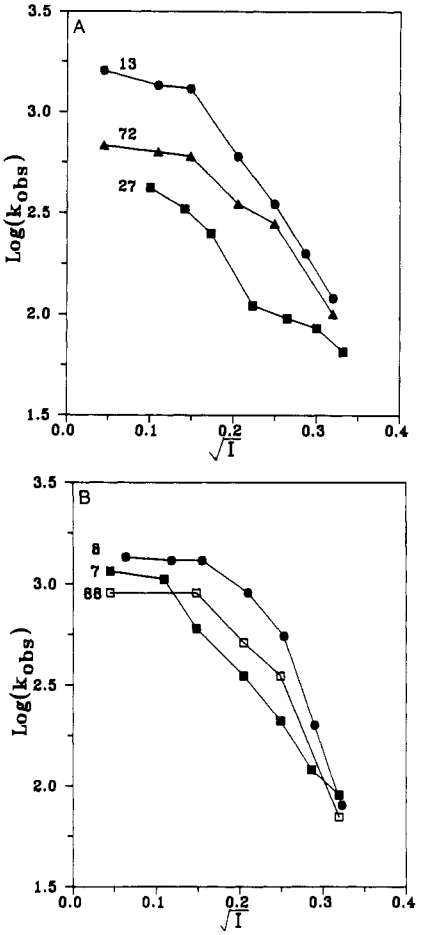



FIGURE 4: Ionic strength dependence of electron transfer from $Ru(bpy)_2(dcbpy)$–cytochrome *c* derivatives to plastocyanin. $k_{obs}$ is plotted as a function of ionic strength for derivatives modified at lysines 13 (●), 27 (■), and 72 (▲) (A) and lysines 7 (■), 8 (●), and 86 (□) (B). The conditions were the same as described in Figure 1 except that a plastocyanin concentration of 30 $\mu$M was used in all measurements and sodium chloride was added to adjust the ionic strength.

will be carried out to address this question. Finally, the $Ru^{II}(bpy)_2(dcbpy)$–lysine group bears the same net charge, +1, as the native lysine amino group, and all of the derivatives were found to bind tightly to plastocyanin at low ionic strength. The large size of the ruthenium group (9-Å radius; Figure 5) could of course alter the geometry of the complex with plastocyanin and hence the rate of electron transfer.

The reaction between cytochrome *c* and plastocyanin has been studied by using a number of different approaches. Augustin et al. (1983) and Rush et al. (1988) carried out stopped-flow kinetics measurements of the reaction of plastocyanin with cytochrome *c* derivatives modified at individual lysines with carboxydinitrophenyl (CDNP). The second-order rate constants for the derivatives modified at lysines 13 and 27 were decreased by about 50% compared to that of native cytochrome *c*, while the derivatives modified at other lysines were decreased to a smaller extent. These studies indicated that the reaction domain for plastocyanin was centered at the top part of the heme crevice and involved lysines 13, 27, 86, and 25. King et al. (1985) and Chapman et al. (1984) carried out NMR and kinetic studies indicating that the interaction domain on plastocyanin involved the "negative patch" consisting of Tyr 83 and the four acidic residues 42–45. The rate of reverse intracomplex electron transfer from plastocyanin to cytochrome *c* was measured to be 87 $s^{-1}$, from which the rate of the forward electron-transfer reaction was estimated to be 4800 $s^{-1}$. Geren et al. (1983) used the water-soluble

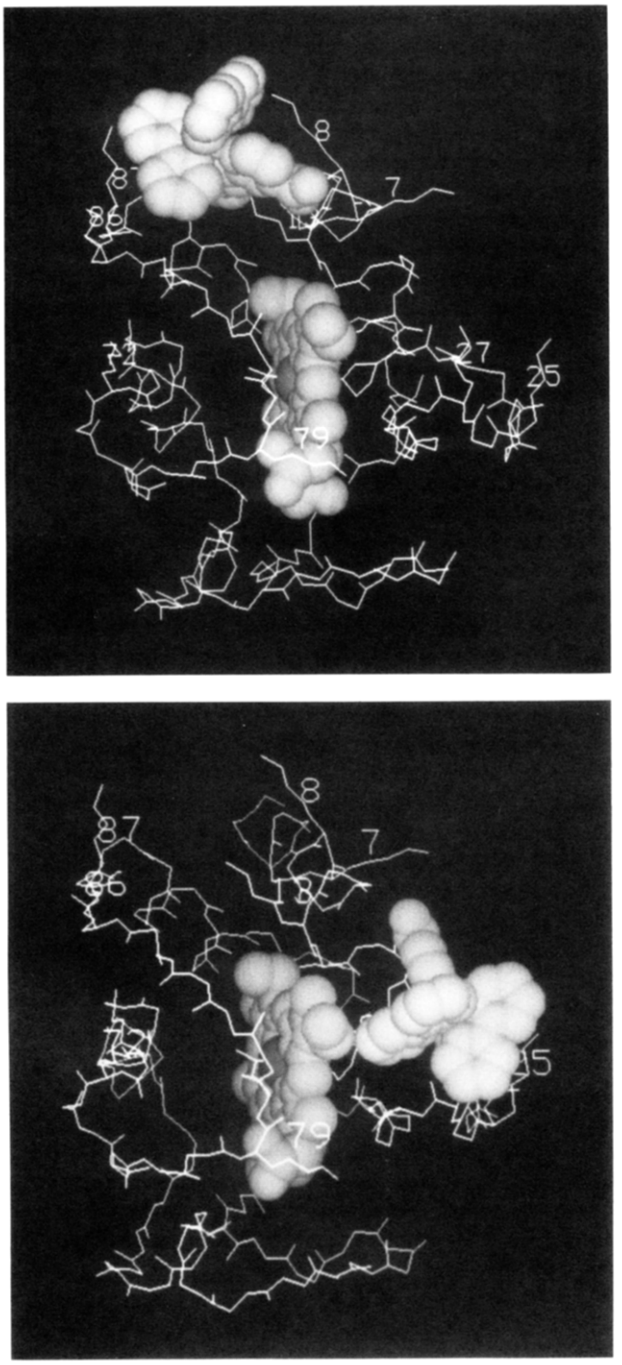



FIGURE 5: Molecular models of $Ru^{II}(bpy)_2(dcbpy)$–cytochrome *c* derivatives modified at lysines 13 (top) and 27 (bottom). The peptide backbone of bonito cytochrome *c* is viewed from the front of the heme crevice with the side chains of lysines 7, 8, 13, 25, 27, 72, 79, 86, and 87 numbered (Protein Data Bank File: 1cyc; M. Kakudo). The $Ru^{II}(bpy)_2(dcbpy)$ group and the heme group are represented by CPK space-filling models. The models were obtained on a Silicon Graphics W-4D25G workstation using BIOSYM Insight software. The Ru-$(bpy)_2(dcbpy)$–lysine group may assume different orientations from the one shown due to the flexibility of the lysine side chain.

carbodiimide EDC to form a 1:1 cross-linked complex between cytochrome *c* and plastocyanin. The complex was inactive with cytochrome oxidase and the cytochrome $bc_1$ complex, indicating that the heme crevice domain of cytochrome *c* was the site of cross-linking. Peerey and Kostič (1989) have recently purified four different 1:1 cross-linked complexes between cytochrome *c* and plastocyanin and studied their electron-transfer properties by pulse radiolysis. The rate of electron transfer in the electrostatic complex between the two native proteins was found to be 1050 $s^{-1}$, but no intracomplex electron transfer was detected in any of the cross-linked complexes. The authors suggested that protein reorientration might be necessary for intracomplex electron transfer.

It is interesting that the largest rate constant for intracomplex electron transfer was observed for the $Ru(bpy)_2$-(dcbpy)–cytochrome *c* derivative modified at lysine 13 at the top of the heme crevice, while the smallest was for the derivative modified at lysine 27 at the right side of the heme crevice. These two lysines appear to be located at the center of the interaction domain as judged by the effects of CDNP modification on the second-order rate constant measured at high ionic strength (Augustin et al., 1983). The derivative modified at lysine 72 outside the interaction domain had a $k_{et}$ value of 800 $s^{-1}$, similar to that of native cytochrome *c*, 1050 $s^{-1}$ (Perrey & Kostič, 1989). One possible interpretation of these results is that the bulky $Ru^{II}(bpy)_2(dcbpy)$ group might alter the geometry of the complex between cytochrome *c* and plastocyanin and hence the rate of electron transfer. In the complex with native cytochrome *c*, electrostatic interactions with lysines 7, 8, and 13 may cause plastocyanin to bind toward the top of the heme crevice with a geometry that is not optimal for rapid electron transfer. The bulky $Ru^{II}(bpy)_2(dcbpy)$ group on the lysine 13 derivative could then cause plastocyanin to bind toward the lower part of the heme crevice in a more favorable orientation for electron transfer (Figure 5). This interpretation is consistent with the large $k_{et}$ values for the derivatives modified at lysines 7 and 8, which are also located at the top of the heme crevice. In contrast, a ruthenium group on lysine 27 would effectively block access to the lower part of the heme crevice (Figure 5) and cause plastocyanin to bind at the top of the heme crevice. This could account for the small rate of electron transfer observed for the lysine 27 derivative. These interpretations are based on the idea that the ruthenium group alters the geometry of the complex and hence the electronic coupling term for electron transfer from the heme group to the copper atom. It is also possible that the ruthenium group could alter the nuclear reorganization energy for electron transfer, either directly or indirectly by altering the geometry of the complex. The ruthenium group on a heme crevice lysine would be expected to alter the solvation at the interface between the two proteins, and this could affect the solvent contribution to the reorganization energy (Meade et al., 1989). Studies are planned to address this question by measuring the temperature dependence of the rate constants.

Increasing the ionic strength led to a significant increase in $k_{et}$ for both the lysine 13 and lysine 27 derivatives. This suggests that conformations even more favorable for rapid electron transfer are formed at high ionic strength for these derivatives. However, increasing ionic strength did not affect the intracomplex rate constant of the lysine 72 derivative or of native cytochrome *c* (Peerey & Kostič, 1989). Increasing ionic strength has also been observed to increase the rate of intracomplex electron transfer between cytochrome *c* and cytochrome *c* peroxidase (Hazzard et al., 1987b). In another interesting parallel to the present results, the replacement of yeast cytochrome *c* lysine 13 with isoleucine resulted in a significant increase in the rate of electron transfer to cytochrome *c* peroxidase (Hazzard et al., 1988b). Thus, in two separate systems, interaction with lysine 13 appears to lead to a binding geometry that is not optimized for maximal rates of intracomplex electron transfer.

## ACKNOWLEDGMENTS

The molecular models shown in Figure 5 were obtained by using atomic coordinates of bonito cytochrome *c* provided by the Protein Data Bank (File 1cyc; M. Kakudo).

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# Rotational Dynamics of Chloroplast ATP Synthase in Phospholipid Vesicles†

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ABSTRACT: The rotational dynamics of the purified dicyclohexylcarbodiimide-sensitive $H^+$-ATPase (DSA) reconstituted into phospholipid vesicles and of the DSA coreconstituted with the proton pump bacteriorhodopsin were examined by using the technique of time-resolved phosphorescence emission anisotropy. The phosphorescent probe erythrosin isothiocyanate was used to covalently label the γ-polypeptide of DSA before reconstitution. Rotational correlation times were measured under a variety of conditions. The rotational correlation time was independent of the viscosity of the external medium but increased significantly as the microviscosity of the membrane increased. This indicates the rotational correlation times are a measure of the enzyme motion within the membrane. The activation energy associated with the rotational correlation time is 8-10 kcal/mol. At 4 °C, the correlation time, typically ~100-180 μs, was unaffected by the addition of substrates and the presence of a membrane pH gradient. Therefore, molecular rotation of the DSA does not appear to play an important role in enzyme catalysis or ion pumping.

The dicyclohexylcarbodiimide-sensitive $H^+$-ATPase (DSA)[1] from spinach chloroplasts is composed of a soluble portion, coupling factor 1 ($CF_1$), and a membrane component, $CF_0$. The entire protein complex consists of at least nine different polypeptide chains and is responsible for coupling the synthesis of ATP to the flow of protons across the thylakoid membrane. DSA can be isolated, purified, and reconstituted into phospholipid vesicles. The reconstituted enzyme catalyzes ATP-$P_i$ exchange (Winget et al., 1977; Pick & Racker, 1979). While the exact mechanism for catalysis by the coupling factor is still not known, activation and catalysis clearly involve conformational changes in the $CF_1$ portion of the enzyme (Farron & Racker, 1970; Girault & Galmiche, 1977; Wagner & Junge, 1980; Bruist & Hammes, 1982; Pick & Finel, 1983; Leckband & Hammes, 1987; Shapiro & McCarty, 1988). Most of the evidence for these changes has been obtained by studying $CF_1$ in solution, and the dynamic motions of the more complex membrane-reconstituted DSA have not been thoroughly investigated.

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[1] Abbreviations: DSA, dicyclohexylcarbodiimide-sensitive $H^+$-ATPase; $CF_1$, coupling factor 1 of DSA; $CF_0$, coupling factor 0 of DSA; bR, bacteriorhodopsin; DPH, 1,6-diphenyl-1,3,5-hexatriene; ErITC, erythrosin isothiocyanate; EDTA, ethylenediaminetetraacetic acid; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine.

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