# Rapid Charge Separation and Bathochromic Absorption Shift of Flash-Excited Bacteriorhodopsins Containing 13-Cis or All-Trans Forms of Substituted Retinals†

H.-W. Trissl*,‡ and W. Gärtner§

*Department of Biology/Chemistry, Schwerpunkt Biophysik, University of Osnabrück, D-4500 Osnabrück, FRG, and Biozentrum, CH-4056 Basel, Switzerland*

*Received February 6, 1986; Revised Manuscript Received October 7, 1986*

ABSTRACT: Bacteriorhodopsins (BRs) containing retinal, 13-ethylretinal, 13-methoxyretinal, and 13-demethylretinal were investigated flash spectroscopically and photoelectrically. In all preparations, bathochromic absorption bands (indicating K intermediate formation) occurred within less than 5 ns although the analogue BRs, due to the chemical structure of the retinal compounds, were in an invariant 13-cis or all-trans form. The lifetimes of these K states varied over 6 orders of magnitude. Photoelectric measurements demonstrated that a rapid charge separation process occurred within less than 100 ps for all preparations. The magnitude and the direction of the charge separation process were nearly the same for all BRs, except of that of 13-demethylretinal-containing BR, which under identical conditions exhibited a 3 times larger amplitude. Thus, an early charge separation connected with a red shift of the absorption maximum is the common consequence of light absorption. This process takes place, regardless of whether the chromophore is originally bound to the protein in the all-trans configuration with the "anti" geometry for the Schiff base group or in the 13-cis configuration with the "syn" geometry for the Schiff base group [Harbison, G. S., Smith, S. O., Pardoen, J. A., Winkel, C., Lugtenburg, J., Herzfeld, J., Mathies, R., & Griffin, R. G. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 1706–1709]. The K intermediates form as the result of the disruption of salt bridges between the protonated Schiff bases and their unprotonated counterions for the cis or trans states, respectively, and the subsequent association of the Schiff base groups to another protonated group. On the basis of the generally accepted mechanism, a possible explanation for the exclusively trans-coupled proton translocation and the malfunction of the cis cycle is discussed.

Bacteriorhodopsin (BR)[1] converts light energy into electrochemical energy by actively pumping protons across a membrane. Essential for this function is one molecule of retinal, covalently bound as a protonated Schiff base to a lysine residue of the apoprotein (Birge, 1981; Stoeckenius & Bogomolni, 1982). Further interaction between the binding site and the retinal molecule results in a strongly red-shifted absorption band, compared to the $\lambda_{max}$ of the protonated Schiff base of retinal and butylamin. This difference was termed "opsin shift" (Nakanishi et al., 1980).

In the dark-adapted state of BR the thermodynamic equilibrium between the 13-cis and all-trans isomers of retinal is approximately one to one (Oesterhelt & Hess, 1973; Oesterhelt et al., 1973; Pettei et al., 1977; Sperling et al., 1977; Maeda et al., 1977). Both isomeric forms show only slightly different absorption maxima. Continuous illumination shifts this equilibrium completely to the all-trans form ($\lambda_{max}$ 568 nm). Upon flash excitation, both isomeric forms of BR undergo photochemical reactions producing a series of spectroscopically distinguishable intermediate states. These reactions re-form the original BR state within a few milliseconds and are called the cis and the trans photocycle. It is widely believed that the primary photophysical event involves a trans–cis or cis–trans isomerization, respectively (Stoeckenius & Bomolni, 1982). The active transport of protons is found only for the trans cycle but not for the cis cycle (Ohno et al., 1977; Lozier et al., 1978; Fahr & Bamberg, 1982).

One of the earliest events of the trans cycle is the formation of the bathochromic K intermediate within 5 ps (Zinth et al., 1985), which lives for several microseconds. The K formation is accompanied by an all-trans to 13-cis isomerization of the retinal chromophore (Zinth et al., 1985; Polland et al., 1984; Hsieh et al., 1981; Braiman & Mathies, 1982). By this process, about 30% of the energy of the photons is stored (Birge & Cooper, 1983). As a consequence of the isomerization, the protonated Schiff base group moves away from its counterion (Zinth et al., 1985; Birge & Cooper, 1983; Warshel, 1979; Honig et al., 1979a,b; Shulten & Tavan, 1978), which most likely is the negatively charged carboxy group of an aspartic acid, as was detected by FTIR spectroscopy (Engelhard et al., 1985). The electrogenic character of this reaction has been demonstrated by photoelectric experiments (Trissl, 1983, 1985; Groma et al., 1984), and calorimetric studies have shown that this charge separation accounts for more than 50% of the total energy stored (Birge & Cooper, 1983).

Much less is known about the cis cycle. The available data indicate the formation of long-living bathochromic species (Tokunaga et al., 1976; Ohno et al., 1977). The fate of these forms (time resolution of several microseconds) demonstrates the absence of any hypsochromic M-like cis intermediates (Sperling et al., 1977). The thermodynamic ground state of the cis cycle is repopulated in milliseconds without pumping protons (Sperling et al., 1977; Fahr & Bamberg, 1982). The reason for the absence of proton-pumping activity is not known. Extended knowledge of the primary reactions occurring in the cis cycle may therefore give valuable information on the primary events of the pumping mechanism.

Under this aspect we reconstituted the apoprotein bacterioopsin (BO) with various retinal analogues modified at

† This work was financially supported by the Deutsche Forschungsgemeinschaft, Sonderforschungsbereich 171, Teilprojekt A1.

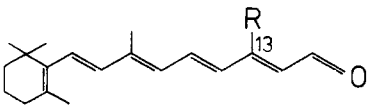
‡ University of Osnabrück.

§ Biozentrum.

[1] Abbreviations: BR, bacteriorhodopsin; $BR_{DA}$ and $BR_{LA}$, dark- and light-adapted bacteriorhodopsin; BO, bacterioopsin; FTIR, Fourier transform infrared spectroscopy; HPLC, high-pressure liquid chromatography.

0006-2960/87/0426-0751$01.50/0 

position 13 and measured their spectroscopic and electric activities. Similar to unmodified retinal, the analogues bind to the opsin via a protonated Schiff base and also show strongly red-shifted absorption bands. The reconstituted BRs have the advantage over native BR of displaying an isomeric composition of the chromophore in the binding site, which is not affected upon illumination (Gärtner & Oesterhelt, 1987; Gärtner et al., 1983). The structural formulas of the retinals are




| | |
|---|---|
| retinal | R = $CH_3$ |
| 13-ethylr. | R = $C_2H_5$ |
| 13-methoxyr. | R = $OCH_3$ |
| 13-demethylr. | R = H |

We report here on the formation of bathochromic intermediates, which are complete in less than 5 ns. This process is common to all BRs studied, regardless of whether they are in the 13-cis or all-trans conformation. Furthermore, all these analogues showed a very fast charge separation of the same polarity. On the basis of these data, we develop a model that explains why only illumination of the all-trans form of BR leads to pumping of protons.

## Materials and Methods

*Retinal Analogues and Preparations.* The synthesis of 13-demethylretinal is described elsewhere (Gärtner et al., 1980). 13-Ethylretinal was obtained from Drs. H.-J. Bestmann and P. Ermann, University of Erlangen, FRG (Ermann, 1982). 13-Methoxyretinal was provided by H. Hopf and K. Natsias, University of Braunschweig, FRG (Natsias & Hopf, 1982). *all-trans*-Retinal was purchased from Fluka. All chemicals and reagents were of optimal purity. The retinal compounds were tested for purity prior to use by HPLC (Gärtner et al., 1980). The halobacterial strains JW5 and M9 were provided by Prof. D. Oesterhelt, MPI Biochemie, Martinsried, FRG. Purple membranes and white membranes were prepared according to the procedure described for purple membranes (Oesterhelt & Stoeckenius, 1974). BRs containing retinal (retinal BR), 13-demethylretinal (13-demethyl BR), 13-ethylretinal (13-ethyl BR), or 13-methoxyretinal (13-methoxy BR) were reconstituted by the addition of 25–28 nmol of the retinal compounds, dissolved in 5–10 μL of 2-propanol, to an aqueous suspension (1 mL) of 32 nmol of white membranes. The extent of reconstitution of the light-adapted forms was followed spectroscopically with the following molar extinction coefficients at the wavelengths indicated: BR, $\epsilon_{568}$ = 60 000 $M^{-1}$ $cm^{-1}$ (Oesterhelt & Hess, 1973; Rehorek & Heyn, 1979); 13-methoxy BR, $\epsilon_{515}$ = 40 000 $M^{-1}$ $cm^{-1}$ (Gärtner & Oesterhelt, 1987); 13-demethyl BR, $\epsilon_{565}$ = 60 000 $M^{-1}$ $cm^{-1}$ (Gärtner et al., 1983); and 13-ethyl BR, $\epsilon_{560}$ = 60 000 $M^{-1}$ $cm^{-1}$ (Oesterhelt et al., 1987).

*Laser Flash Spectroscopy.* Spectroscopic measurements were carried out in a 1 × 1 cm quartz cuvette. The optical density of the different samples ranged between 0.3 and 0.67 at their $\lambda_{max}$. The excitation source was a Q-switched and frequency-doubled Nd YAG laser with a pulse duration of 12 ns (full width at half-maximum). The samples were excited at a wavelength of 532 nm with an energy of 1.5 mJ/$cm^2$. For fast-flash spectroscopy ($t < 20$ μs), the measuring light source was a xenon flash with a duration of ~100 μs. Monochromatic light was obtained with interference filters ($\Delta\lambda \approx 7$ nm) at the wavelengths given in the figures. The energy of measuring flashes was on the order of 0.7 mJ/$cm^2$. The excitation laser flash was delivered to the sample about 60 μs after the beginning of the discharge. Absorbance changes were detected with a fast-responding (<1 ns) photodiode (EG&G, type FND 100). For measurements in the submicrosecond range, the working resistance was 100 Ω. It was ac-coupled to a 50-Ω preamplifier (Ortek, type 535; voltage gain 10; bandwidth dc 180 MHz). The value of the coupling capacitor was chosen sufficiently small so that the discharge profile of the xenon flash was differentiated close to zero and sufficiently large so that the kinetics of absorbance changes were not affected by the resulting RC decay. This was checked experimentally by sending rectangular light pulses from a LED (light emitting diode) to the measuring diode. For measurements around 1 μs, a working resistance of 1 kΩ was chosen, and a custom-built preamplifier with adjustable offset was applied (input resistance 100 kΩ; gain 50; bandwidth dc 7 MHz).

*Photoelectric Measurements.* To get comparable relative amplitudes of the photoelectric responses from the different reconstituted BRs, the same batch of white membranes was used when the reconstitution was started. The different samples were then treated always the same.

Two different sets of photoelectric measurements were done. In one set, with a moderate time resolution (~10 ns), the membranes were electrically oriented as described earlier (Trissl, 1985). The excitation source was the Q-switched and frequency-doubled Nd YAG laser. In the other set of experiments with greater time resolution (<1 ns), the measuring cell was considerably reduced in size. This cell was constructed into an SMA connector and had a coaxial design. Each of the two metal electrodes had a diameter of 3 mm. They were separated by a Teflon spacer at a distance of 0.1 mm. One plate was constructed as a Pt grid (Trissl, 1985) and the other one as a solid Pt plate. The excitation source for these experiments was a mode-locked and frequency-doubled Nd YAG laser delivering pulses of 30-ps duration. The photovoltage signals were amplified by a 50 Ω amplifier (Nucletudes, type 60-20-1; voltage gain 30 dB; bandwidth 10 MHz–6 GHz) before they were recorded on a 1-GHz oscilloscope (Tektronix, type 7104). Single shots on the oscilloscope screen were digitized by a Nocticon camera (Thomson CSF, Model 1150) modified for signal averaging. All measurements were carried out at room temperature, 21 ± 1 °C. The samples were handled only under very dim light.

## Results

*Flash Spectrometric Measurements.* When light-adapted (LA) BR, reconstituted from retinal and BO, was subjected to short laser flashes, an increase of the optical density at 630 nm (downward deflections in Figure 1) was observed. It appeared with the time course of the laser flash (Figure 1a; LA) and indicates the formation of the bathochromic K intermediate. The signal decayed with a time constant of 1.5 μs to a level above the base line (Figure 1e; LA), indicating the formation of the blue-shifted L intermediate ($\lambda_{max}$ 550 nm). These measurements with BR containing 100% *all-trans*-retinal confirm similar ones of other laboratories (Lozier et al., 1975).

As mentioned, $BR_{DA}$ contains an approximately 1:1 mixture of *all-trans*- and 13-*cis*-retinal (Oesterhelt et al., 1973). Experiments with such a sample contain informations on the 13-cis photocycle. In order to minimize conversion into the LA state by the measuring light and the excitation flash, only

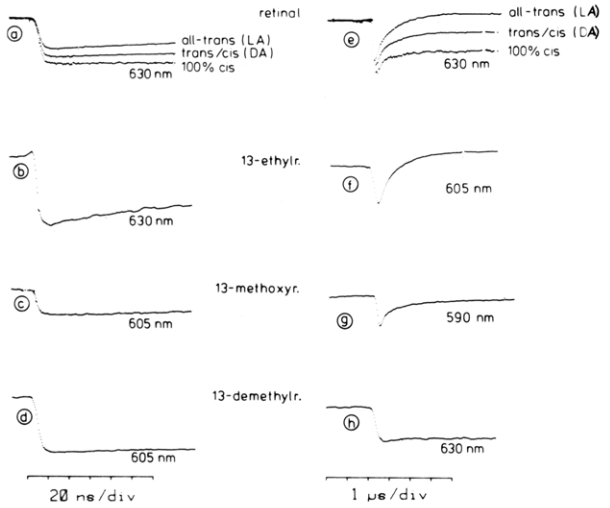



FIGURE 1: Absorbance changes, $\Delta I/I$, in the nano- and microsecond time range of BRs, reconstituted with different retinal analogues, induced by laser flashes (532 nm; 12 ns; 1.5 mJ/cm$^2$). Downward deflections indicate less light falling onto the photodiode and correspond to an increase in absorbance. The wavelength quotations in the figure indicate the wavelength of the measuring light for the given sample and the given time range. If not otherwise indicated, the samples were in the dark-adapted state, DA (light-adapted = LA).

two traces were averaged. The corresponding absorbance changes are shown in panel a (DA) and panel e (DA) of Figure 1. In order to obtain a 100% 13-cis signal, 50% of the LA signal was substracted from the DA signal, and the result was multiplied by 2. In contrast to the all-trans signal, the main absorbance of the 100% cis signal change was stable for more than 5 µs (Figure 1e; 100% cis). This bathochromic intermediate is reported to decay with a half-time of 37 ms (Sperling et al., 1977). The small negative overshoot in this trace might be due to a substraction artifact or the presence of slightly more than 50% trans isomer in the DA samples (Harbison et al., 1984).

Excitation of 13-ethyl BR (30% cis and 70% all-trans) also showed a fast-appearing red-shifted intermediate (Figure 1b). The signal decayed with a time constant of 0.55 µs to a blue-shifted form similar to the L intermediate in BR as indicated by positive values of the light intensity (Figure 1f).

A fast-absorbance increase was also found for 13-methoxy BR (30% 9,13-cis,cis and 70% 13-cis) (Figure 1c). However, the signal decayed to the base line in a biphasic manner with time constants of 0.5 µs (67% of the amplitude) and 5 µs (33%) (Figure 1g), suggesting that the faster phase is due to the 13-cis form and the slower phase is due to the 9,13-cis,cis form. At longer time scales, absorption changes of less than 15% of the early bathochromic shift were observed (data not shown; BR′ in Table I). This result is in contrast to the measured spectroscopic behavior of BR, where the L formation is obvious by the crossing of the base line (Figure 1e). It indicates that the K intermediate of 13-methoxy BR directly re-forms the parent BR state.

The absorbance changes of 13-demethyl BR (85% 13-cis and 15% all-trans) in the two time ranges are shown in panels d and h of Figure 1, respectively. There was only a very small decaying phase observed with a time constant of approximately 0.5 µs, which correlates with the amount of all-trans isomer in the sample (Table I). More than 80% of the remaining part of this bathochromic state decayed back to BR with a single exponential time constant of 0.4 s as shown in Figure 2. The recorded "single-shot" trace demonstrates also for the 13-demethyl cis probe the lack of a hypsochromic intermediate. There was no difference between LA and DA samples.

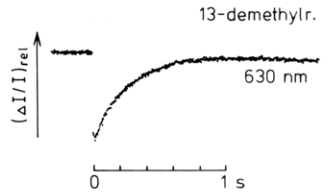



FIGURE 2: Single-shot absorbance change, $\Delta I/I$, at a wavelength of 630 nm of 13-demethyl BR measured on a slow time scale. To avoid photoaccumulation of products by multiple photon absorption, the measuring light was reduced to 5 µW/cm$^2$. Excitation condition was as in Figure 1. Recording bandwidth dc was 300 kHz.

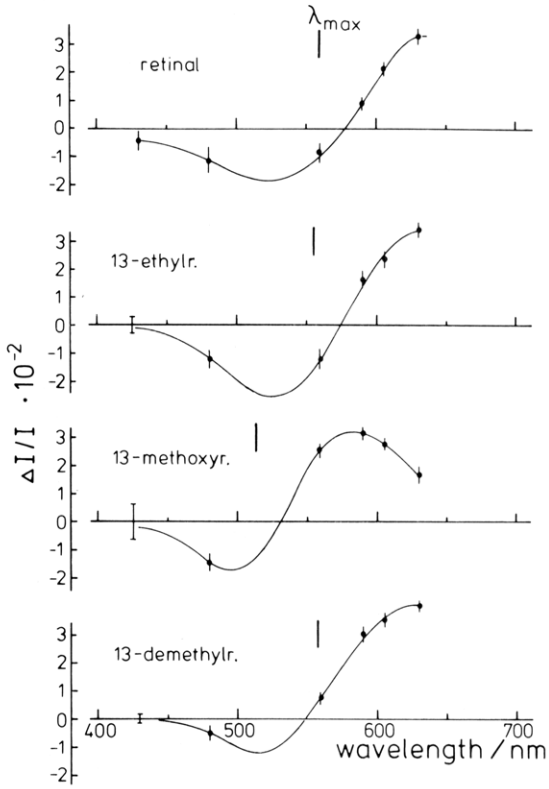



FIGURE 3: Difference spectra of BRs reconstituted with different retinal analogues as indicated. Readings were made at the end of a 12-ns flash (532 nm; 1.5 mJ/cm$^2$). The relative changes, like those in Figure 1, were normalized to the optical density and the molar extinction coefficient of each sample. The vertical bars indicate the absorption maxima of the dark-adapted samples.

The absorbance changes in Figure 1 were depicted without calibration scale, since the light intensity falling onto the measuring diode varied with the different samples and wavelengths used. To achieve comparable data, the measured intensity changes were divided by the absolute intensity, yielding relative absorbance changes. These, in turn, were normalized to the optical density and the molar extinction coefficient of a given sample at the excitation wavelength (the excitation energy was held constant throughout). From these normalized absorbance changes read 10 ns after the onset of the laser flash, the difference spectra in Figure 3 were derived.

The spectra given in Figure 3 illustrate the influence of an electronically modified substituent at position 13 on the opsin shift, as well as on the extent of the red shift during the formation of K intermediates. Whereas the opsin shift is similar for 13-*cis*- and *all-trans*-retinal BR, 13-ethyl BR, and 13-demethyl BR, it is smaller by 45 nm for 13-methoxy BR ($\Delta$18 kJ/mol). The red shift upon K formation, however, is practically the same for all analogue BRs studied, indicating the same relative energy differences between the ground and the K states.

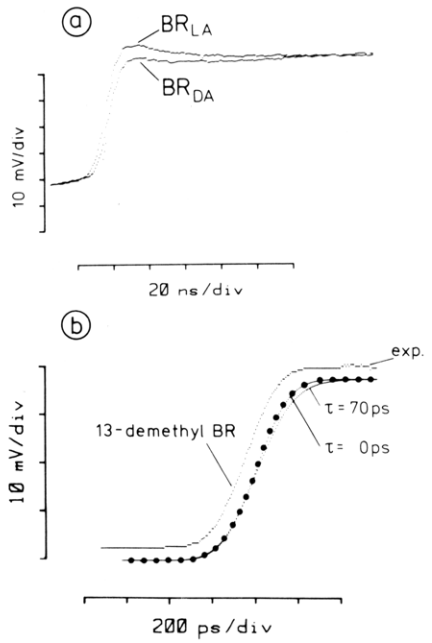



FIGURE 4: (a) Photovoltage from electrically oriented retinal BR in the dark-adapted (DA) and light-adapted (LA) state, evoked by 12-ns laser flashes at 532 nm. Excitation energy was 0.5 mJ/cm$^2$. (b) (Upper trace) Time course of the rising phase of the photovoltage from 13-demethyl BR evoked by a single 30-ps flash at 532 nm. (Lower traces) Best fit of the photovoltage by the calculated response function of the oscilloscope as described by an error function ($\tau$ = 0 ps). For comparison, the experimental data are marked by thick points. The dotted smooth curve to the right represents the convolution of monoexponential charge separation kinetics with the time constant $\tau$ = 70 ps with the instrumental response function.

*Photoelectric Measurements.* The BRs reconstituted from different analogues and BO were vectorially oriented by an electric field (Trissl, 1985; Keszthelyi, 1980). This allowed us to study their flash-induced electrical activity by measuring the corresponding photovoltage. Two sets of experiments were carried out: In one set with a 10-ns time resolution the constant energy of the Q-switched Nd YAG laser was utilized to evaluate quantitatively the photovoltage amplitude. Regardless of their 13-cis or all-trans configuration, all samples displayed a photovoltage of the same polarity, rising with the time course of the laser flash (Figure 4a). The relative amplitudes, normalized to the optical densities and the molar extinction coefficients, are listed in Table I. Except for 13-demethyl BR, which was 3 times as active, all other analogues displayed similar amplitudes.

In order to reveal the electric activity connected with the K formation from 13-*cis*-retinal BR, we measured the photovoltage from DA and LA samples under otherwise identical conditions. As seen in Figure 4a, the two traces were almost indistinguishable (accuracy ±5%).

In a second set of photoelectric experiments with higher time resolution, the membranes were oriented in a microcoaxial cell (Deprez et al., 1986) and excited by 30-ps flashes. Again, all analogues displayed a photovoltage within the rise time of the apparatus. The 13-demethyl BR gave the largest signal. A typical "single-shot" photovoltage record of this latter preparation is depicted in Figure 4b (experimental trace).

The rate constant of the electrogenic reaction was estimated by fitting the data with the response function of the apparatus convoluted with assumed monoexponential charge separation kinetics (Deprez et al., 1986). The response of the oscilloscope to a pure step function was an error function, as proved by test pulses. Also, the photovoltage from 13-demethyl BR could be well described by this function (Figure 4b; lower part). A significant deviation from this best fit occurred when the time constant of the charge separation was chosen to be 70 ps (instead of 0 ps in the case of a pure step function) (Figure 4b; lower part). This demonstrated that the kinetics of the charge separation in 13-demethyl BR and in the other analogue BRs is faster than or equal to 70 ps. A more detailed analysis of such data can be found in Deprez et al. (1987).

## Discussion

Our results obtained from $BR_{LA}$ and $BR_{DA}$ and from analogue BRs reconstituted from the various retinal analogues can be summarized: (i) Light absorption caused in all BRs the formation of bathochromically absorbing intermediates (K states). (ii) This process is accompanied by a fast charge separation with a polarity opposite to the direction of the proton translocation (Trissl, 1983; Keszthelyi, 1980; Fahr et al., 1981). (iii) Both processes occur with striking similarity in BRs containing the chromophore in the 13-cis or all-trans configuration. These findings will be discussed together with the proton-pumping activity of the analogues BRs (Gärtner et al., 1983; Oesterhelt et al., 1987; Tavan et al., 1985a).

*Photocycles of BR Analogues.* The comparable shifts of the absorption maxima between the all-trans and 13-cis BRs and their K intermediates suggests that the Schiff base converts to a similar microenvironment during this transition, which is characterized by the movement of the Schiff base from a charged to an uncharged group. Possibilities of how this can be accomplished by different protonation states of the protein will be discussed later.

Whereas the formation of the K intermediates was invariably fast for all preparations, the stability varied strongly over 6 orders of magnitude, depending on the particular substituent. In cases where the decays of $K_{cis}$ and $K_{trans}$ could be distinguished, the $K_{trans}$ decayed faster [compare Oesterhelt et al. (1987)].

13-Ethyl BR behaved most similar to BR in the formation of the photocycle intermediates, in agreement with recently published proton translocation activities (Tavan et al., 1985a; Oesterhelt et al., 1987). The conversion of its $K_{trans}$ intermediate into the L intermediate was slightly faster than the corresponding process of BR (Figure 1 and Table I). This might be due to an increased steric hindrance between the bulky ethyl group at position 13 and the binding site, evolving during the K formation, which leads to the accelerated conversion into the L state.

13-Methoxy BR, carrying a substituent of comparable size as the ethyl group, also showed a submicrosecond decay of its K form. Obviously, a similar steric repulsion as in 13-ethyl BR accelerates this decay. Due to the exclusive presence of the 13-cis isomer, only the direct re-formation of the parent BR state occurs. Such a reduced photocycle appears to be characteristic for the 13-cis isomers. The high abundance of the 13-cis isomer of 13-methoxy BR is attributed to $\pi$-electron interactions that force the methoxy group into a coplanar arrangement with the polyene chain (Tavan et al., 1985a).

By considering the absorption maxima of the ground states of the photocycle intermediates of 13-methoxy BR, it appears as if the *absolute* energy level of a photocycle is controlled by the substituent, resulting for 13-methoxy BR in a comparable blue shift of the absorption spectra of the BR state and the K state. Once a defined cis/trans distribution is formed under the control of a particular substituent, the *relative* energy barriers, i.e., the sequence of the intermediates and the kinetics of the photocycle, are predominantly governed by forces derived from the protein.

The high amount of the 13-cis isomer in 13-demethyl BR

Table I: Summary of Main Experimental Data on Chemically Modified BRs[a]

| chromophore | cis/trans ratio | photocycle and known intermediates | relative amplitude of fast charge separation | $H^+$-pump activity |
|---|---|---|---|---|
| retinal | | | | |
| DA | 50% cis/50% trans | | −0.9 | 0.5 |
| LA | 100% trans | $BR \xrightarrow{<5ns} K \xrightarrow{1.5\,\mu s} L \rightarrow$ | −1.0 | 1.0 |
| | 100% cis | $BR \xrightarrow{<5ns} K_1, K_2 \xrightarrow{ms} BR$ | −0.8 | 0 |
| 13-ethylretinal | 30% cis/70% trans | $BR \xrightarrow{<5ns} K \xrightarrow{0.5\,\mu s} L \rightarrow$ | −0.8 | 0.7 |
| 13-methoxyretinal | 30% 9,13-cis,cis/70% 13-cis | $BR \xrightarrow{<5ns} K_1 \xrightarrow{0.5\,\mu s} BR'$ ($K_2$, 5 μs) | −0.7 | 0.02 |
| 13-demethylretinal | 85% cis/15% trans | $BR \xrightarrow{<5ns} K \xrightarrow{0.4\,s} BR$ | −3.3 | 0.16 |

[a] Summary of experimental data available for light- and dark-adapted BR and for the analogue BRs. In the cases of mixed isomeric forms, only the main intermediates of the photocycle are considered. In the fourth column, the negative sign of the charge separation indicates a movement of a positive charge away from its counterion (opposite to the transmembrane $H^+$ dislocation) (Trissl, 1983). The numbers in the second column are taken from Gärtner et al. (1983, 1986) and Fendler et al. (1987).

seems to result from altered sterical interactions with the binding site. Following the general behavior of cis-derived photointermediates, the 13-demethyl $K_{cis}$ also reconverts directly back to the ground state of the photocycle. The lack of a methyl group at position 13, however, increases the stability of $K_{cis}$ up to 400 ms (Figure 2), emphasizing the importance of steric effects of the $C_{13}$ substituent.

The charge separation signal of 13-demethyl BR was 3 times as large as that of the other BRs (Table I). It is not likely that this would mean a 3 times larger distance traveled by the protonated Schiff base group away from its counterion, since a larger distance between the two charges in the K state would cause a more strongly red-shifted absorption maximum (Blatz et al., 1972), which is not observed (Figure 3). Additionally, the extent of mobility is likewise restricted by the aliphatic side chain of Lys-216 for both retinal compounds, retinal and 13-demethylretinal. This explanation would suggest that it is more likely the parent ground state that has a different conformation than the K intermediate. For a more detailed interpretation, it has to be taken into account that the absolute amplitude of the fast photovoltage is composed of vectorial components of the charge separation, which are perpendicular to the membrane plane, the degree of bond rotations, the inclination of the plane of rotation (Figure 5), and contributions of changes of the permanent dipole moments. Although the electronic structures of retinal and 13-demethyl retinal are very similar, meaning that the mentioned electrogenic processes can be assumed to be also very similar, the lack of information on the quantities does not allow for a clear-cut description.

As already mentioned, no basic differences in the absorption properties and the excitation behavior between the 13-cis and all-trans BRs could be detected, and in dark-adapted BR both isomers appear in a nearly 1:1 ratio (thermodynamic equilibrium). It should be mentioned that the cis and the trans BR states differ also in the configuration of the Schiff base $C{=}N^+$ bond (Harbison et al., 1984). Unfortunately, the bond rotations upon K formation are only partially known for the cis state. Even for the trans state diverging experimental results exist: Some groups vote for a solely 13–14 isomerization (Smith et al., 1986), whereas others claim that also the 14–15 bond rotates during K formation (Gerwert & Siebert, 1986).

To explain the similar absorption spectra of the corresponding K states (Tokunaga et al., 1976) and the observation that cis cycle converts to the trans cycle via a bathochromic intermediate (Tokunaga et al., 1976; Sperling et al., 1977), one may assume that the electronic situation of both K forms in respect of the interactions between the protonated Schiff base and the undissociated group is comparable. It may even be that both K forms adopt similar conformations. This is not unlikely if one considers not only plain cis/trans photoisomerizations to take place but more generally simultaneous distortions of several bonds of the polyene chain. This was already proposed for the 13-cis geometry of the K intermediates formed in trans cycle. The resulting conformations of such "likewise" distorted isomerizations may resemble each other more strongly than their respective parent configurations (Hsieh et al., 1981; Braiman & Mathies, 1982). On the basis of these arguments, the contributions of different protein states of the isomeric chromophore forms allow an explanation of the experimental data.

*Schemes for the Primary Events in Cis and Trans Cycles.* If the detected electrical signals are interpreted as charge separations due to photochemically induced bond rotations of the retinal molecule, an important conclusion can be drawn from the experimental observation that all preparations displayed the same orientation of the charge separation: The simple interpretation of a photoisomerization of 13-cis BRs just reverse to that of all-trans BRs cannot hold since this should lead to opposite polarities. Such a simple forward/backward isomerization process can be excluded, even if the charge separation is assumed to represent only a part of the electric signal, which may be composed of several electric processes.

One can neither definitely rule out that the charge separation at the Schiff base is superimposed by a larger electrogenic reaction within the polyene chain, which could have the same direction for both isomeric forms and thus cover the former, nor can one distinguish if the retinal molecule compensates the motion of the Schiff base group leading to a further electrical signal (i.e., change of the retinal's dipole moment). Nonetheless, we want to interprete our data with the simplest assumption, namely, that the photovoltage is due to the charge separation connected with the breakage of a salt bridge at the Schiff base site.

In Figure 5 we present and discuss schemes that make use of four groups $A_1$–$A_4$ having various protonation states (Figure 5). The groups $A_1$–$A_4$ could be assigned to the aspartic acid residues identified by FTIR spectroscopy (Engelhard et al., 1985). However, it could as well be that one group is a tyrosine residue, as was recently proposed (Hamamoto et al., 1984; Rothschild et al., 1986). Since our schemes shall reflect only

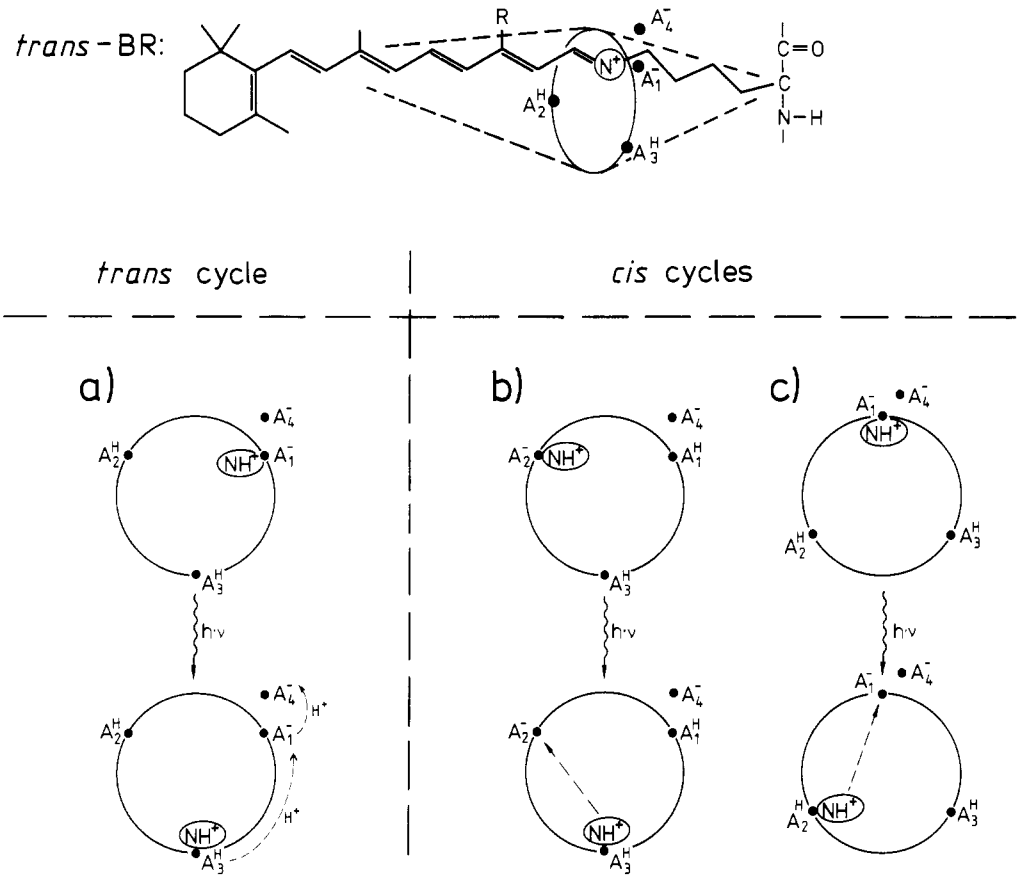



FIGURE 5: (Upper part) Schematic drawing of the binding site of *all-trans*-retinal. It is assumed that in all BRs and their K intermediates the ionone ring and the peptide bond of the lysine residue are fixed. This would mean that the elongated shape of the retinal–lysine backbone is preserved and that the degree of freedom of the Schiff base group is limited to a circle perpendicular to the legth axis, as indicated by the dashed double cone. (Lower part) Views along the length axis of the retinal's polyene chain: Possible arrangements of $NH^+$ with respect to counterions in cis and trans BRs and their corresponding K intermediates. Further explanations are given in the text.

principle interactions between the Schiff base and the protein and their changes caused by the transition from BR to K, no exact assignment of the groups $A_1$–$A_4$ is necessary.

The following aspects shall be covered by the model: (i) The trans and the cis forms convert rapidly to spectrally only slightly different bathochromic K intermediates (Tokunaga et al., 1976; Sperling et al., 1977). (ii) Light absorption causes in both isomeric forms a charge separation of comparable amplitude and the *same* polarity. (iii) This polarity is opposite to the direction of proton transport through the protein (Trissl, 1983; Keszthelyi, 1980; Fahr et al., 1981). (iv) Only the trans but not the cis cycle displays light-driven proton pumping (Ohno et al., 1977; Fahr & Bamberg, 1982; Fendler et al., 1987).

Generally, retinal binding by light-activated proteins exhibits the following properties: The retinal molecules are bound via a protonated Schiff base to the $\epsilon$-amino group of a lysine residue. The bathocrhomic shift of the absorption spectra of various protein states, like the photocycle intermediates of BR, K and O, is due to either the removal of the counterion away from the positively charged Schiff base, as was demonstrated by model compounds (Blatz et al., 1972), or the protonation of the counterion (Fischer & Oesterhelt, 1980). In both cases the net positive charge on the polyene chain of retinal increases (Tavan et al., 1985b). Furthermore, since the prrotein backbone does not significantly move during the primary isomerization reactions and the position of the retinal's ring system and the peptide bond of lysine remain essentially unaltered, the space for movements of the $C{=}N^+$ group induced by isomerizations is restricted to a circle that is nearly perpendicular to the length axis of the polyene chain (Figure 5, top).

As shown in Figure 5, we have located four groups in various protonation states in the vicinity of the Schiff base group. They serve either as negative counterions for the positive charge at the $C{=}N^+$ bond or as "switching elements" for the first steps of proton pumping (Schulten & Tavan, 1978). We propose two possible arrangements of $A_1$–$A_4$, which allow explanation of both the same direction of the early charge displacement and the different proton pumping activities. In one case, the binding environment for *all-trans*- and for 13-*cis*-retinal is different due to different counterions: $A_1^-$ in the all-trans and $A_2^-$ in the 13-cis form (see Figure 5a,b). Residues $A_2$ in (Figure 5a) and $A_1$ in (Figure 5b), which occupy the respective remote positions to the protonated Schiff base, are assumed to be protonated. In the other case (Figure 5c), the $C{=}N^+$ group of 13-cis BR and of all-trans BR interact with the same counterion, $A_1^-$. Absorption of light in all cases leads to a disruption of the salt bridge with $A_1^-$ and $A_2^-$, respectively, explaining the bathochromic shift of the primary photoproducts.

In cases a and b of Figure 5, this process places the $C{=}N^+$ group in the neighborhood of the undissociated group $A_3^H$, whereas in case c of Figure 5 the $C{=}N^+$ group moves toward $A_2^H$. This would explain the comparable size and the *same* direction of the charge separation for all three cases. It is worth mentioning that all mechanisms shown in Figure 5 are in accordance with spectroscopic measurements at low temperatures, which show that a transition of the cis form into the trans cycle occurs via a bathochromic cis intermediate

(Sperling et al., 1977; Tokunaga et al., 1976).

In the case of the trans cycle, the positive charge of the Schiff base nitrogen near $A_3^H$ destabilizes the binding of this proton and allows its transfer to $A_1^-$, forming the L intermediate. The blue-shifted absorption band of the L form is explained by the formation of a new salt bridge between the $C{=}N^+$ group and the now deprotonated residue $A_3^-$. It becomes obvious from the dashed lines in the trans cycle of Figure 5a that the proton at $A_1$ can be transferred further to $A_4^-$, thereby supporting the nucleophilic attack of $A_3^-$ on the proton of the $C{=}N^+$ group, which in the M form is removed from the Schiff base.

The essential point of our proposal is the facile explanation of the direction of proton pumping opposite to the primary charge separation. Additionally, the schemes offer a possible explanation for the inefficiency of the cis cycle to pump protons. Since the group $A_1^H$ is already protonated, there is no other way for the chromophore than to convert back to the $BR_{dark}$ ground state (dashed arrows in Figure 5; cis cycle, b). Accordingly, no hypsochromic intermediate is found in the cis cycle (Sperling et al., 1977). In the alternative arrangement (Figure 5c) of $A_1$, $A_2$, and $A_3$ for the cis cycle, the same group, $A_1^-$, serves as counterion for the cis and trans isomers (Figure 5, cis cycle, c). However, different primary isomerization steps turn the Schiff base group close to $A_2^H$, which is located at the bottom. Again, there is no proton-accepting group close enough to $A_2^H$, so that the chromophore converts directly back to the ground state without proton transfer.

In summary, the proposed schemes explain qualitatively our experimental observations and the different proton-pumping capabilities. Detailed models on the primary photochemistry will require more structural data of the particular chromophore configurations and the binding site. The principal features proposed in our schemes should be met by further, more refined models.

ACKNOWLEDGMENTS

We thank Drs. E. Baker and J. Huebner for critically reading the manuscript.

**Registry No.** Hydrogen ion, 12408-02-5.

REFERENCES

Birge, R. R. (1981) *Annu. Rev. Biophys. Bioeng. 10*, 315–354.
Birge, R. R., & Cooper, T. M. (1983) *Biophys. J. 42*, 61–69.
Blatz, P. E., Mohler, J. H., & Navangul, H. V. (1972) *Biochemistry 11*, 848–855.
Braiman, M., & Mathies, R. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 403–407.
Deprez, J., Trissl, H.-W., & Breton, J. (1987) *Proc. Natl. Acad. Sci. U.S.A. 83*, 1699–1703.
Engelhard, M., Gerwert, K., Hess, B., Kreutz, W., & Siebert, F. (1985) *Biochemistry 24*, 400–407.
Ermann, P. (1982) Ph.D. Thesis, University of Erlangen, FRG.
Fahr, A., & Bamberg, E. (1982) *FEBS Lett. 140*, 251–253.
Fahr, A., Läuger, P., & Bamberg, E. (1981) *J. Membr. Biol. 60*, 51–62.
Fendler, K., Gärtner, W., Bamberg, E., & Oesterhelt, D. (1987) *Biochim. Biophys. Acta* (in press).
Fischer, U. C., & Oesterhelt, D. (1980) *Biophys. J. 31*, 139–146.
Gärtner, W., & Oesterhelt, D. (1987) *Eur. J. Biochem.* (in preparation).
Gärtner, W., Hopf, H., Hull, W. E., Oesterhelt, D., Scheutzow, D., & Towner, P. (1980) *Tetrahedron Lett. 21*, 347–350.
Gärtner, W., Towner, P., Hopf, H., & Oesterhelt, D. (1983) *Biochemistry 22*, 2637–2644.
Gerwert, K., & Siebert, F. (1986) *EMBO J. 5*, 805–811.
Groma, G., Szabo, J., & Varo, G. (1984) *Nature (London) 308*, 557–558.
Hamamoto, J. H., Dupuis, P., & El-Sayed, M. A. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 7083–7087.
Harbison, G. S., Smith, S. O., Pardoen, J. A., Winkel, C., Lugtenburg, J., Herzfeld, J., Mathies, R., & Griffin, R. G. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 1706–1709.
Honig, B., Ebrey, T. G., Callender, H. R., Dinur, U., & Ottolenghi, M. (1979a) *Proc. Natl. Acad. Sci. U.S.A. 76*, 2503–2507.
Honig, B., Dinur, U., Nakanishi, K., Balogh-Nair, V., Gawinowicz, M. A., Arnaboldi, M., & Motto, M. G. (1979b) *J. Am. Chem. Soc. 101*, 7084–7086.
Hsieh, C. L., Nagumo, M., Nicol, M., & El-Sayed, M. A. (1981) *J. Phys. Chem. 85*, 2714–2717.
Keszthelyi, L. (1980) *Biochim. Biophys. Acta 598*, 429–436.
Lozier, R. H., Bogomolni, R. A., & Stoeckenius, W. (1975) *Biophys. J. 15*, 955–962.
Lozier, R. H., Niederberger, W., Ottolenghi, M., Sivorinovski, G., & Stoeckenius, W. (1978) In *Energetics and Structure of Halophilic Microorganisms* (Caplan, S. R., & Ginzburg, M., Eds.) Elsevier Biomedical, Amsterdam and New York.
Maeda, A., Iwasa, T., & Yoshizawa, T. (1977) *J. Biochem. (Tokyo) 82*, 1599–1604.
Nakanishi, K., Balogh-Nair, V., Arnaboldi, M., Tsujimoto, K., & Honig, B. (1980) *J. Am. Chem. Soc. 102*, 7945–7947.
Natsias, K., & Hopf, H. (1982) *Tetrahedron Lett. 23*, 3673–3676.
Oesterhelt, D., & Hess, B. (1973) *Eur. J. Biochem. 37*, 316–326.
Oesterhelt, D., & Stoeckenius, W. (1974) *Methods Enzymol. 31a*, 666–678.
Oesterhelt, D., Meentzen, M., & Schuhmann, L. (1973) *Eur. J. Biochem. 40*, 453–463.
Oesterhelt, D., Gärtner, W., Vogel, J., Maurer, R., & Schneider, S. (1987) *Biochemistry* (submitted for publication).
Ohno, K., Takeuchi, Y., & Yoshida, M. (1977) *Biochim. Biophys. Acta 462*, 575–582.
Pettei, M. J., Yudd, A. P., Nakanishi, K., Henselman, R., & Stoeckenius, W. (1977) *Biochemistry 16*, 1955–1959.
Polland, H.-J., Franz, M. A., Zinth, W., Kaiser, W., Kölling, E., & Oesterhelt, D. (1984) *Biochim. Biophys. Acta 767*, 635–639.
Rehorek, M., & Heyn, M. P. (1979) *Biochemistry 18*, 4977–4983.
Rothschild, K. J., Roeppe, P., Ahl, P. L., Earnest, T. N., Bogomolni, R. A., Das Gupta, S. K., Mullikan, C. M., & Herzfeld, J. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 347–351.
Schulten, K., & Tavan, P. (1978) *Nature (London) 272*, 85–86.
Smith, S. O., Hornung, I., van der Stein, R., Pardoen, J. A., Braiman, M. S., Lugtenburg, J., & Mathies, R. A. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 967–971.
Sperling, W., Carl, P., Rafferty, C. N., & Dencher, N. (1977) *Biophys. Struct. Mech. 3*, 79–94.
Stoeckenius, W., & Bogomolni, R. A. (1982) *Annu. Rev. Biochem. 51*, 587–616.
Tavan, P., Schulten, K., Gärtner, W., & Oesterhelt, D. (1985a) *Biophys. J. 47*, 349–355.
Tavan, P., Schulten, K., & Oesterhelt, D. (1985b) *Biophys. J. 47*, 415–430.

Tokunaga, F., Iwasa, T., & Yoshizawa, T. (1976) *FEBS Lett. 72*, 33–38.
Trissl, H.-W. (1983) *Biochim. Biophys. Acta 723*, 327–331.
Trissl, H.-W. (1985) *Biochim. Biophys. Acta 806*, 124–135.
Warshel, A. (1979) *Photochem. Photobiol. 30*, 285–290.
Zinth, W., Nuss, M. C., Polland, H. J., Franz, M. A., & Kaiser, W. (1985) in *Spectroscopy of Biological Molecules* (Alix, A. J. P., Bernard, L., & Manfait, M., Eds.) pp 325–331, Wiley, Chichester, New York, Brisbane, Toronto, and Singapore.

# Laser Flash Photolysis Studies of Electron Transfer between Ferredoxin–NADP$^+$ Reductase and Several High-Potential Redox Proteins†

Anjan K. Bhattacharyya, Terrance E. Meyer, Michael A. Cusanovich, and Gordon Tollin*
*Department of Biochemistry, University of Arizona, Tucson, Arizona 85721*
*Received April 29, 1986; Revised Manuscript Received October 1, 1986*

ABSTRACT: Complex formation and the kinetics of electron transfer between ferredoxin–NADP$^+$ reductase (FNR) and two structurally homologous acidic 4Fe–4S high-potential ferredoxins (HiPIP's) from *Ectothiorhodospira halophila* (HP1 and HP2) and two structurally homologous cytochromes $c_2$ from *Paracoccus denitrificans* and *Rhodospirillum rubrum* (PC2, and RC2, respectively) have been investigated by gel filtration and laser flash photolysis techniques. Gel filtration studies indicated that complex formation occurred between $FNR_{ox}$ and $HP1_{ox}$ or $HP2_{ox}$ at low ionic strength (10 mM) and that the complexes were completely dissociated at high ionic strength (310 mM). Laser flash photolysis using lumiflavin as the reductant demonstrated that both free $HP1_{ox}$ and $HP2_{ox}$ reacted primarily with the anionic form of fully reduced lumiflavin ($LFH^-$), whereas FNR was unreactive. Second-order rate constants of $1 \times 10^6$ and $0.8 \times 10^6$ $M^{-1}$ $s^{-1}$ were obtained for these reactions at 10 mM ionic strength. Increasing the ionic strength to 310 mM resulted in an approximately 1.5-fold increase in the rate constant. Inclusion of stoichiometric amounts of $FNR_{ox}$ into the reaction mixture at low ionic strength led to a 2.5-fold increase in the rate constants. The reaction of 5-deazariboflavin semiquinone (5-dRf·) with the oxidized HiPIP's was also investigated by laser flash photolysis. Second-order rate constants of $3.0 \times 10^8$ $M^{-1}$ $s^{-1}$ (HP1) and $2.5 \times 10^8$ $M^{-1}$ $s^{-1}$ (HP2) were obtained for the free proteins at 10 mM ionic strength. Under the same conditions, 5-dRf· reacted with free $FNR_{ox}$, resulting in the formation of the neutral protein-bound semiquinone (FNR·), with a second-order rate constant of $6 \times 10^8$ $M^{-1}$ $s^{-1}$. The reduction kinetics of 1:1 $FNR_{ox}$–$HiPIP_{ox}$ complexes at $I$ = 10 mM were also investigated. In the case of the $FNR_{ox}$–$HP1_{ox}$ complex, reduction by 5-dRf· led to the initial rapid formation of both FNR· and $HP1_{red}$. A subsequent, slower intracomplex electron-transfer reaction from FNR· to $HP1_{ox}$ was observed to occur with a first-order rate constant of 23 $s^{-1}$. The reduction kinetics of the $FNR_{ox}$–$HP2_{ox}$ complex using 5-dRf· were similar to those of the $FNR_{ox}$–$HP1_{ox}$ complex. However, the limiting first-order rate constant corresponding to the intracomplex reaction between FNR· and $HP2_{ox}$ was much smaller ($k$ = 4 $s^{-1}$). Gel filtration experiments indicated that complex formation between $FNR_{ox}$ and $PC2_{ox}$ or $RC2_{ox}$ occurred at low ionic strength (10 mM) and dissociation was observed at high ionic strength (310 mM). Lumiflavin semiquinone (LfH·) reacted with both cytochromes, and second-order rate constants of $5.5 \times 10^7$ and $7.9 \times 10^7$ $M^{-1}$ $s^{-1}$ were obtained with $PC2_{ox}$ and $RC2_{ox}$, respectively. The reactivity of the cytochromes with LfH· within the complex, i.e., in the presence of stoichiometric amounts of $FNR_{ox}$ at low ionic strength, remained unchanged, suggesting that access of LfH· to the heme was not influenced by the presence of FNR. The 5-dRf· reaction with the oxidized free cytochromes gave second-order rate constants of $6 \times 10^8$ and $9 \times 10^8$ $M^{-1}$ $s^{-1}$ for $PC2_{ox}$ and $RC2_{ox}$ at $I$ = 10 mM. The 5-dRf· reaction at low ionic strength (10 mM) with the 1:1 $FNR_{ox}$ complexes with both cytochromes resulted in the initial rapid formation of both reduced cytochrome and FNR·. This was followed by a slower first-order intracomplex electron transfer from FNR· to the oxidized cytochromes. First-order rate constants of ~700 and ~400 $s^{-1}$ were obtained for $PC2_{ox}$ and $RC2_{ox}$, respectively. At high ionic strength, the reaction of FNR· with oxidized $PC2_{ox}$ was second order ($k = 5 \times 10^6$ $M^{-1}$ $s^{-1}$), consistent with the observed dissociation of the complex. The results of these experiments are interpreted in terms of possible structural features of the complexes formed between FNR and the redox protein electron acceptors. The physiological FNR–Fd complex is the only one in which access to the electron-transfer site is diminished, probably because it is located within the protein–protein interaction domain. The intracomplex electron-transfer reactions within the nonphysiological complexes are slower than expected on the basis of the thermodynamic driving force (redox potential difference), most likely as a result of nonoptimal orientations between redox centers, and larger distances over which electron transfer must occur.

In previous work from this laboratory, we have reported on the kinetics of electron-transfer reactions between various physiological and nonphysiological donors and acceptors in an attempt to elucidate the factors involved in determining the rates of electron transfer to and within electrostatically stabilized protein–protein complexes (Przysiecki et al., 1985; Bhattacharyya et al., 1986; Ahmad et al., 1982; Simondsen

† This work was supported in part by NIH Grants AM 15057 (to G.T.) and GM 21277 (to M.A.C.).

0006-2960/87/0426-0758$01.50/0 