Steric Interaction between the 9-Methyl Group of the Retinal and Tryptophan 182 Controls 13-*cis* to *all-trans* Reisomerization and Proton Uptake in the Bacteriorhodopsin Photocycle†

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ABSTRACT: The hypothesis was tested whether in bacteriorhodopsin (BR) the reduction of the steric interaction between the 9-methyl group of the chromophore *all-trans*-retinal and the tryptophan at position 182 causes the same changes as observed in the photocycle of 9-demethyl-BR. For this, the photocycle of the mutant W182F was investigated by time-resolved UV−vis and pH measurements and by static and time-resolved FT-IR difference spectroscopy. We found that the second half of the photocycle was similarly distorted in the two modified systems: based on the amide-I band, the protonation state of D96, and the kinetics of proton uptake, four N intermediates could be identified, the last one having a lifetime of several seconds; no O intermediate could be detected; the proton uptake showed a pronounced biphasic time course; and the pK_a of group(s) on the cytoplasmic side in N was reduced from 11 in wild type BR to around 7.5. In contrast to 9-demethyl-BR, in the W182F mutant the first part of the photocycle does not drastically deviate from that of wild type BR. The results demonstrate the importance of the steric interaction between W182 and the 9-methyl group of the retinal in providing tight coupling between chromophore isomerization and the late proton transfer steps.

In the light-driven proton pump bacteriorhodopsin (BR;[1] reviewed by Mathies et al., 1991; Lanyi, 1992, 1993; Oesterhelt et al., 1992; Rothschild, 1992; Ebrey, 1993; Krebs & Khorana, 1993) the isomerization of the *all-trans* retinal, which is bound to the protein via a protonated Schiff base, to the 13-*cis* geometry upon absorption of a photon initiates the two important proton transfer steps, i.e., deprotonation of the Schiff base with concomitant protonation of D85, and reprotonation of the Schiff base with concomitant deprotonation of D96. The thermal reisomerization of the retinal to *all-trans*, the reprotonation of D96, and the deprotonation of D85 complete the cyclic photoreaction. Although reaction models using branched and parallel pathways (Eisfeld et al., 1993; Song et al., 1994) cannot be excluded, a linear cycle which includes back-reactions appears sufficient to describe most experimental and mechanistic aspects of the photoreaction (Lanyi, 1992, 1993): BR → K ↔ L ↔ M_1 → M_2 ↔ N ↔ O → BR. It is assumed in this model that the proton of the Schiff base is the one being transported. Deprotonation and reprotonation of the Schiff base occur during the L → M_1 and M_2 → N transition, respectively, and the retinal is reisomerized to the *all-trans* geometry in the O intermediate. Additionally, multiple sequential M intermediates have been identified at low temperature (Friedmann et al., 1994), and for the D96N mutant an M state after M_2 has been identified which has a protein conformation similar to the N state (Sasaki et al., 1992). As in any ion pump, changes of binding constant and of accessibility of groups mediating the transport control the unidirectional ejection and uptake of ions. Thus, the isomerization of the chromophore induces pK_a changes of the Schiff base and of the carboxyl groups D85 and D96 (Brown et al., 1993; Cao et al., 1993). In addition, it changes the accessibilty of the Schiff base from the extracellular to the cytosolic side (Ames & Mathies, 1990; Váró & Lanyi, 1991; Kataoka et al., 1994; Tittor et al., 1994). This molecular switch is thought to occur during the irreversible or quasi-irreversible M_1 → M_2 transition. Therefore, in order to understand the molecular mechanism of bacteriorhodopsin, it is important to gain information on the chromophore−protein interaction that mediates the functionally important alterations of the protein. We have shown earlier that for the visual pigment rhodopsin the steric interaction between the 9-methyl group of the chromophore and the protein is of upmost importance for activation of the signaling state of the pigment: removal of the methyl group inhibits signal transduction (Ganter et al., 1989). For the bacterial sensory pigment sR-I the importance of the 13-methyl group has been stressed (Yan et al., 1991). Therefore, similar steric interactions could be functionally important in bacteriorhodopsin also. In a recent investigation, we have shown that removal of the 9-methyl group greatly alters the

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[1] Abbreviations: FT-IR, Fourier transform infrared; UV−vis, ultraviolet−visible; BR, bacteriorhodopsin; 9-H-BR, 9-demethylbacteriorhodopsin; HOOP, hydrogen out-of-plane; W182F, bacteriorhodopsin mutant tryptophan-182 → phenylalanine; WT, wild type.

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photocycle (Weidlich et al., 1995). A drastic increase of the lifetime of the N intermediate and decoupling of chromophore reactions from proton transfer steps were observed. Based on the three-dimensional model of bacteriorhodopsin (Henderson et al., 1990) and recent FT-IR results providing evidence for the steric interaction between the 9-methyl group and W182 (Yamazaki et al., 1995), we have suggested that it is the perturbation of this steric interaction that causes these alterations. If this is correct, one would expect that introduction of an amino acid smaller than tryptophan at position 182 would produce similar effects.

By performing time-resolved UV−vis and proton uptake experiments and static and time-resolved FT-IR investigations, we compare in this article the photoreactions of 9-demethyl BR and the mutant W182F. Whereas differences between the two systems are observed for the early part of the photocycle (up to M_1), the later part is similarly influenced in the following respects: four N species can be distinguished, the last one exhibiting a lifetime of several seconds, very little O accumulates, and the proton uptake kinetics show a characteristic biphasic behavior. We conclude that, whereas proton transfer from the cytosol to the Schiff base is facilitated, the reisomerization of the chromophore is inhibited, resulting in a photocycle with much longer relaxation time.

MATERIALS AND METHODS

The preparation of purple membranes containing 9-demethylretinal as chromophore has been described previously (Weidlich et al., 1995). The preparation of the bacteriorhodopsin mutant W182F has been published (Yamazaki et al., 1995). Time-resolved UV−vis measurements at selected wavelength and time-resolved pH measurements were performed according to Brown et al. (1994b). In order to avoid the generation of photoproducts by the probing beam, which is a special problem because of the drastically prolonged photocycle (see Results), the intensity of the measuring light was greatly reduced. This forced us to limit the electronic bandwidth. In one series of time-resolved UV−vis experiments using in this case samples suitable for infrared measurements, a shutter was used operating synchronously with the data acquisition. This allowed us to increase the intensity of the probing beam and, correspondingly, the electronic bandwidth. In this way, the earlier part of the photocycle (from 1 μs onward) could be also investigated. The probing beam caused measurable absorbance decrease from 30 to 100 ms after opening of the shutter, depending on the wavelength used. The corresponding time courses were subtracted from the flash-induced signals, reproducing the traces for the later part of the cycle obtained with low measuring intensity and purple membranes in suspension. At the end of the data acquisition, the shutter was closed and the system allowed to relax for 60 s before a new signal was measured. The reproduction of the time course for the later part of the photocycle demonstrates that the state of the samples used for infrared measurements does not influence the observed kinetics. The methods for obtaining static and time-resolved rapid-scan FT-IR spectra from hydrated film samples have been described (Weidlich et al., 1995). Spectral resolution is 2 and 4 cm^{-1} for the static and time-resolved measurements, respectively.

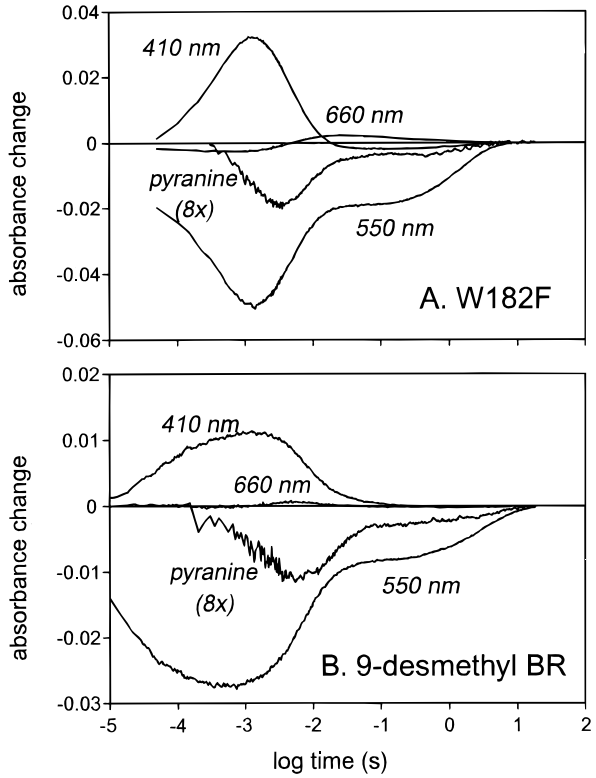

FIGURE 1: Time-resolved abssorbance changes of the mutant W182F and of 9-demethyl-BR at 410 nm (M intermediate), 550 nm (depletion signal), and 660 nm (O intermediate). The net absorbance changes at 457 nm due to the pH indicator dye pyranine are also shown.

RESULTS

Time-Resolved UV−Vis Measurements. Figure 1 compares the transient absorption changes of the mutant W182F and 9-demethyl-BR (9-H-BR) at 660 nm (O intermediate), 550 nm (depletion of initial state), and 410 nm (M intermediate). In addition, the absorption changes of the pH indicator dye pyranine are shown. From the depletion signals, it is clear that in both systems a very long-lived intermediate with an absorbance at 550 nm of 30−40% of the initial state decays only after 10 s in 9-H-BR and 5 s in W182F. In 9-H-BR the efficiency of the photoconversion is reduced by a factor of approximately 5 (Weidlich et al., 1995). Therefore, in order to obtain a sufficient signal/noise ratio, especially for the weak pyranine dye signal, the electronic bandwidth had to be reduced. This caused a distortion in the early part of the rising phase of the signals of 9-H-BR (up to approximately 50 μs). The decay of the signal at 410 nm of W182F is comparable to that of WT BR, and that of 9-H-BR is slightly slowed down. The major (faster) component of the decay of the depletion signal roughly paralles the M decay in both systems. Furthermore, very little, if any, absorbance changes at 660 nm are observed, indicating that negligible amounts of O are accumulated. The dye signal reflects increase (decrease of absorbance) and decrease (increase of absorbance) of proton concentration. The latter, since it is slow, has been shown to reflect the actual uptake process by the protein, whereas the former is delayed with respect to the proton ejection process (Heberle et al., 1994; Alexiev et al., 1995). Similarly

to the decay of the depletion signals, the proton uptake signals exhibit pronounced biphasic behavior in both proteins, with the same time constant as the slow phase of the depletion signal. A difference between the two systems, however, is also evident: whereas for the W182F mutant the fast decay of the proton signal parallels that of the depletion signal, for 9-H-BR the plateau of the proton signal is delayed relative to the depletion signal. Also, the rising phase of the signals at 550 and 410 nm differ in the two systems. As has already been reported by Yamazaki et al. (1995), the effective M rise is considerably slowed down in the mutant and maximum M is only accumulated after approximately 2 ms (350 μs for WT BR). This was attributed to an L/M_1 equilibrium shifted toward L. In 9-H-BR the rise of M, however, is considerably faster than in the mutant (and a corresponding faster phase is observed in the depletion signal). This can be seen even though the electronic bandwidth is reduced. It is important to note that in both systems the final phase of proton uptake which parallels the decay of the long-lived intermediate amounts to approximately 20% at pH 6.4. This fraction is pH dependent. In both systems the decay is somewhat slowed down at pH 7.8 (from 3.8 to 5.5 s for 9-H-BR and from 1.6 to 3.6 s for W182F). More importantly, at the higher pH the amplitude of the slow phase of the proton signal increases from 20% to 60% (data not shown).

In order to better describe the rise of the M state, we repeated the measurements on 9-H-BR with higher light intensities. As mentioned in Materials and Methods, in this case data acquisition has been synchronized with the opening of a shutter for the probing beam. Since we could use a larger electronic bandwidth, we were now able to resolve more clearly two phases in the M rise, the fast phase having a half-time of about 8 μs with an amplitude amounting to approximately 65% of the total amplitude, and a corresponding fast phase has also been observed in the depletion signal at 550 nm (data not shown). This fast phase of M rise was not detected by Yamazaki et al. (1995), probably due to the limited electronic bandwidth they used.

FT-IR Experiments. The photocycle of 9-H-BR had been characterized before by static and time-resolved FT-IR spectroscopy. We performed similar experiments with W182F. The static K and M spectra of the mutant obtained at low temperature had been reported earlier (Rothschild et al., 1989). Our measurements agree with the earlier results and are not reproduced here. Figure 2 compares the L spectra of WT BR, 9-H-BR, and the mutant W182F. The spectra of the wild type BR and the mutant are almost identical. Only the positive lobe of the band at 1730 cm^{-1}, which is caused by an environmental change of protonated D115, is more pronounced in the mutant, as described by Yamazaki et al. (1995). It is not surprising that this residue is influenced by the mutation of W182, since, according to the three-dimensional structure, D115 is very close to W182 (Henderson et al., 1990). The spectrum of 9-H-BR, taken from Weidlich et al. (1995), has already been discussed. Unlike the other two systems, 9-H-BR does not show the typical 11−12 HOOP modes at 960 (negative) and 951 (positive) cm^{-1}. In all three spectra a feature around 750 cm^{-1} is evident which had previously been assigned to tryptophans (Roepe et al., 1988). Figure 3 shows an expanded view of this spectral range. The difference band at 741 (negative) and 746 (positive) cm^{-1} could be assigned

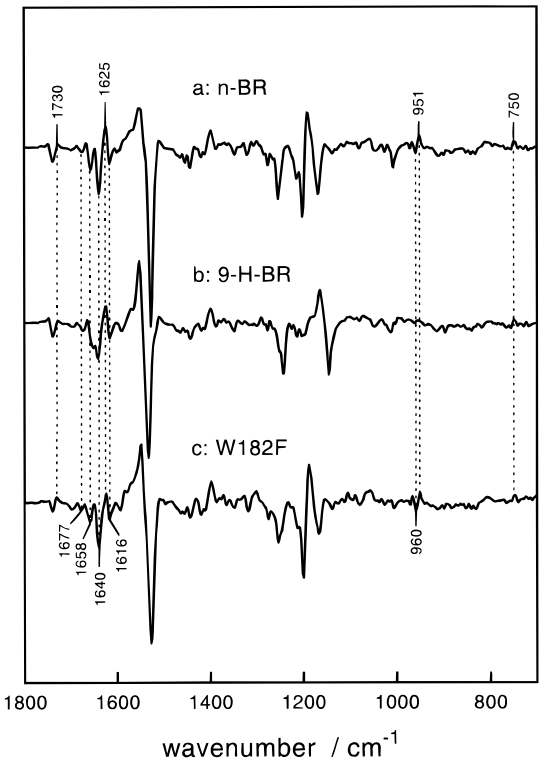

FIGURE 2: Static BR → L FT-IR difference spectra of WT BR (n-BR, a), of 9-H-BR (b), and of the mutant W182F (c) measured at 170 K.

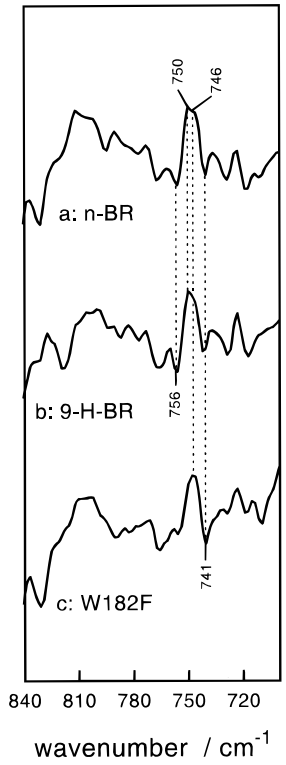

FIGURE 3: Enlarged view of Figure 2, showing the HOOP bands of tryptophan.

to W86, but for the 756−750 cm^{-1} difference band an unequivocal assignment could not be made (Rothschild et al., 1989), although the possibility was discussed that it might be due to W182. Since this difference band is nearly absent in the spectrum of the mutant, our data support this assignment. It should be mentioned that, in contrast to the earlier report in which mutants expressed in *Escherichia coli*

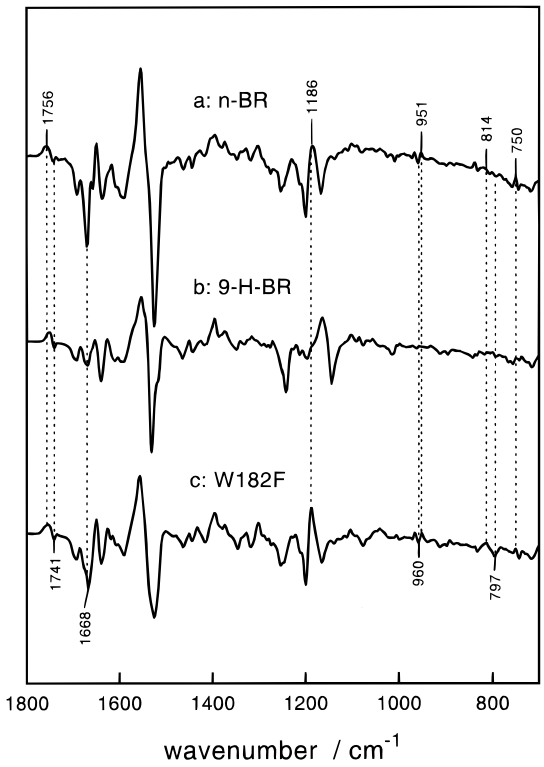

FIGURE 4: Static steady-state FT-IR differeence spectra of WT BR at pH 9 (n-BR, a), showing the BR → M_2/N transition with dominating N contribution, of 9-H-BR at neutral pH (b, BR → N transition), and of the mutant W182F at neutral pH (c, BR → N transition).

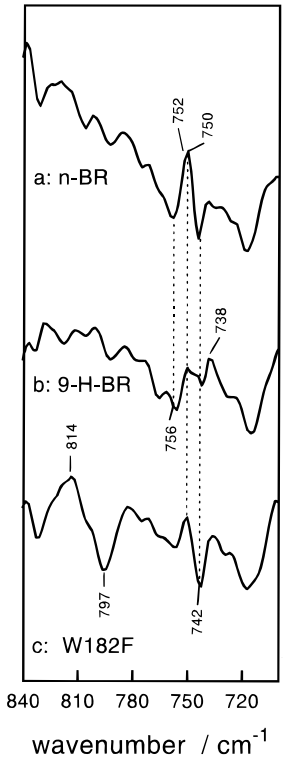

FIGURE 5: Enlarged view of Figure 4, showing the HOOP bands of tryptophan.

had been used, the mutant expressed in *Halobacterium salinarium* shows normal photochemical conversion. In addition, the band appears more clearly in the L spectrum, which had not been shown before. Therefore, the assignment is now unequivocal. The residual band at 756 cm^{-1} in the spectrum of the mutant has been shown to be not caused by a tryptophan vibration (Roepe et al., 1988). It is important to note that the difference band at 756/750 cm^{-1} assigned to W182 is equally present in the spectrum of 9-H-BR.

Steady-state illumination of WT BR at 268 K and pH 7 produces a mixture of mainly M and a small amount of N, whereas, as we have shown previously, illumination of 9-H-BR at this pH generates N. In the wild type protein the amount of N can be made to dominate by increasing the pH to 9 (Fahmy et al., 1993). In Figure 4 the steady-state spectra of WT BR (pH 9, mainly N), 9-H-BR (pH 7, N), and the mutant (pH 7) are compared. It is clear that the mutant spectrum represents an almost pure N spectrum, characterized by the strong fingerprint band at 1186 cm^{-1}, a band due to protonated D85 at 1753 cm^{-1}, and the strong negative amide-I mode at 1668 cm^{-1}. The larger intensity of the positive fingerprint band as compared to that of WT BR indicates that more N is produced. However, the negative band at 1740 cm^{-1} due to deprotonated D96 has approximately the same intensity in the two spectra. This indicates that, as in 9-H-BR, D96 is only partially deprotonated in the N state produced by steady-state illumination. The 11−12 HOOP modes present in the L spectra of WT BR and of the mutant can also be seen in the corresponding N spectra. Again, they are missing in the N spectrum of 9-H-BR. Bands caused by tryptophans around 750 cm^{-1} can also be seen in the N spectra. The extended spectral range reveals now clear differences in the spectra (Figure 5). Since the spectral features observed in the N/M mixture with dominating N are similar to that of low-temperature M (Rothschild et al., 1989), the differences cannot be caused by variable photoproduct compositions. For WT BR, the spectrum is similar to the L spectrum; only the difference band due to W86 is somewhat shifted to higher frequencies. This is mainly due to the positive part which has its peak now at 750 cm^{-1}. In the spectrum of the mutant, similarly to the corresponding L spectrum, the difference band at higher frequencies is again missing, corroborating its assignment to W182. Interestingly, in contrast to the L spectra, removal of the 9-methyl group influences both W182 and W86: the corresponding bands have very low intensities or are even missing in the spectrum of 9-H-BR. In the N spectrum of the mutant a clear difference band is visible at 797 (negative) and 814 (positive) cm^{-1}. This band is not present in the spectra of WT BR and of 9-H-BR. From a comparison with spectra of monosubstituted benzene, we assign this band to the HOOP mode of 5 adjacent hydrogens (Lin-Vien et al., 1991) from the newly introduced phenylalanine. Interestingly, this band is missing in the corresponding L spectrum.

For 9-H-BR, the M → N transition was studied by rapid-scan FT-IR spectroscopy at 263 K. We performed similar experiments for the W182F mutant, shown in Figure 6. The spectrum obtained at 6−17 ms after the flash is very similar to that of 9-H-BR. From the band at 1762 cm^{-1} it can be estimated that about 25% M are present. In contrast to the steady-state spectrum, a clear negative band due to deprotonated D96 is now visible, in addition to the positive band at 1753 cm^{-1} due to protonated D85. However, unlike for 9-H-BR, there is now a negative band amide-I at 1668 cm^{-1}. Its intensity is still considerably lower than that of the corresponding band of N in WT BR. The fully developed band, which appears only in the later spectra, is one of the

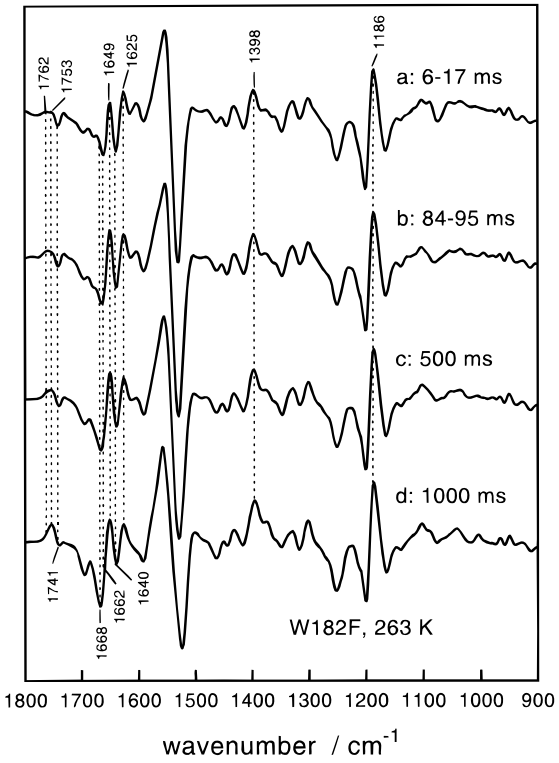

FIGURE 6: Time-resolved rapid-scan FT-IR difference spectra of the mutant W182F measured at 263 K. The respective time ranges of the spectra are given.

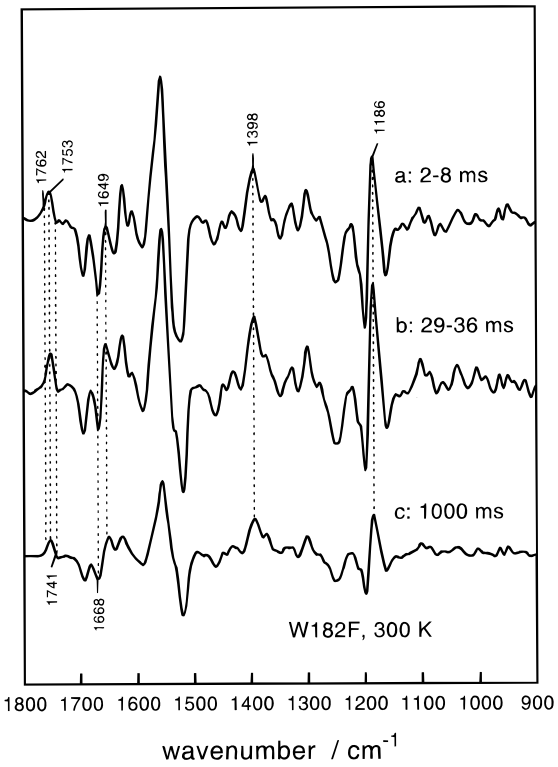

FIGURE 7: Time-resolved rapid-scan FT-IR difference spectra of the mutant W182F measured at 300 K. The respective time ranges are given.

characteristics of N of unmodified BR. Since the intensity at 1762 cm^{-1} is almost unchanged in the spectrum taken 84−95 ms after the flash, little M decay occurs in this time range, as in 9-H-BR. In spite of that, the negative amide-I band at 1668 cm^{-1} is increasing. The spectra taken at 500 and 1000 ms after the flash show the gradual decay of M with a concomitant increase of the fingerprint band at 1186 cm^{-1}, i.e., increase in N. D96 becomes reprotonated in the same time range. The 1000 ms spectrum now shows the full intensity of the negative amide-I band, typical of the N state. It must be noted, however, that after this time, as judged from the intensity of the ethylenic mode, a small fraction of N has already decayed. One could argue that the 6−17 ms spectrum contains, instead of an early N, contributions from L. However, a close inspection of the amide-I bands reveals that the spectral features are rather characteristic of an N-like intermediate. The L difference spectrum (Figure 2) exhibits a small negative band at 1658 cm^{-1}. In the rapid-scan spectrum, the negative band is located at 1662 cm^{-1} and its intensity is considerably larger. Since the presence of approximately 25% M has been deduced for this spectrum, the size of the putative L band would be further reduced. The shift of the negative band from 1668 to 1662 cm^{-1} can be explained by the overlap with the strong negative band at 1658 cm^{-1} present in the M spectra (e.g., Weidlich et al., 1995). The position at 1668 cm^{-1} is only observed after complete M decay. Thus, both the small size and the frequency (1658 cm^{-1}) of the amide-I band in the L spectrum argue against the presence of L in the rapid-scan spectrum. From the time-resolved FT-IR data we can discern, as in the case of 9-H-BR, three N intermediates. We also have performed time-resolved rapid-scan measurements at 300 K (Figure 7). In the first spectrum after the flash accessible to the measurement (2−8 ms), the lack of a negative band at 1740 cm^{-1} demonstrates that D96 is already reprotonated. This is in contrast to 9-H-BR where a slower phase of reprotonation could be discerned. The presence of a shoulder at 1762 cm^{-1} due to protonated D85 in M and the lower intensity of the fingerprint band at 1185 cm^{-1} as compared to that in the 29−36 ms spectrum show that under these conditions (2−8 ms) a small amount of M is still present, in agreement with the time-resolved UV−vis measurements. Thus, the N state of this M/N equilibrium is characterized by an already reprotonated D96.

DISCUSSION

We had described earlier the consequences of removing the 9-methyl group of the retinal on the photocycle (Weidlich et al., 1995). A long-lived N intermediate with a decay time of several seconds was formed, and three N states differing in the protein properties but not in the chromophore structure could be distinguished. No O intermediate could be detected. As compared to WT BR, the extent of photolysis was reduced by a factor of 5 with a 20 ns laser pulse used for excitation. The apparent M amplitude was further reduced by a factor of 3, which had been explained by a fast phase of the M decay. Some aspects of the early part of the photocycle are also influenced. At 80 K, instead of the K photoproduct, an intermediate similar to the KL state was stabilized. Furthermore, at 213 K and high pH, conditions under which normally low-temperature M is obtained, a mixture of L and M was produced. We postulated that the observed alterations are caused by removal of steric interaction between the chromophore and the protein, and suggested that this interaction is between W182 and the 9-methyl group. If this hypothesis were correct, one would expect that reduction in size of the amino acid side chain at position 182 causes similar alterations in the photocycle as removal of the methyl group at position 9 of the retinal. Obviously,

the time-resolved UV−vis data, the time-resolved FT-IR spectra at 263 and 300 K, and the steady-state FT-IR spectra at 268 K in the present work show that the later parts of the photocycle are very similar in the two modified systems. Both show a long-lived N decaying in the time range of several seconds (N decay of 9-H-BR is somewhat slower than that of W182F). Since M completely decays long before N does, it is clear that the M/N equilibrium is interrupted for the later forms of N (Weidlich et al., 1995).

So far, three forms of N could be identified from the FT-IR data at 263 K, the last one being characterized by complete reprotonation of D96. Since no corresponding absorbance changes are resolved in the UV−vis measurements, it can be concluded that the absorption maximum of N is not influenced by the protonation state of D96. Assuming the same classification of the N states as deduced for 9-H-BR, one has to assume that, in order to explain the presence of the negative amide-I band at 1668 cm^{-1}, the transition from N_1 to N_2 is faster than in 9-H-BR. However, it cannot be excluded that in W182F already N_1 exhibits some negative intensity at this position. But otherwise, both systems behave very similarly at 263 K. The presence of 20−30% M in the first two 263 K spectra is explained by the M/N equilibrium prevailing for this time range. This equilibrium also explains the small amount of M present in the 2−8 ms spectrum measured at 300 K. The large amount of N observed at both temperatures, however, demonstrates that there must be an M → N transition considerably faster than in WT BR. This fast decay of M competes with its slow rise, resulting for the mutant and for 9-H-BR in an apparent low M amplitude, which was already deduced from UV−vis measurements.

Proton uptake is biphasic in both systems, the smaller and slower phase occurring concurrently with the N decay. The pH dependence of the two amplitudes suggests that the proton uptake occurs with a pK_a near the pH, resulting first in a proton equilibration of the uptake system with the bulk followed by a shift of this equilibrium to complete reprotonation. This can be represented by the following scheme: $N^{(-1)} + H^+ \leftrightarrow N^{(0)} \rightarrow BR$. Only if the pH is not too far from the pK_a of $N^{(0)}$, the amplitude of $N^{(0)}$ and thus the amplitude of the fast phase of the proton uptake depend on pH: rising or lowering of the pH decreases or increases the fast phase of the proton uptake signal. The slow phase corresponds to the remainder of the reaction, i.e., the $N^{(0)} \rightarrow$ BR transition. The comparison of the proton uptake signals with the time-resolved FT-IR spectra measured at 300 K clearly shows that reprotonation of D96 is decoupled from proton uptake. Thus, one or several additional groups between D96 and the aqueous phase of the cytosolic side are required to explain the data. Similar conclusions based on time-resolved proton uptake and spectral measurements at high pH have been reached previously (Cao et al., 1993b; Brown et al., 1994b). In contrast to WT BR for which the effective pK_a of these group(s) in N has been determined to be around 11 (Zimányi et al., 1993), for N of the two modified systems the pK_a must be around 7.5 as deduced from the increase of the amplitude of the slow phase of the proton uptake signal at pH 7.8. Reprotonation of D96 occurs from these group(s). At 300 K, the fast phase of proton uptake, which is triggered by but still slower than reprotonation of D96, is somewhat slower in 9-H-BR than in the mutant. This may be caused by the different reprotonation times of D96, which is somewhat slower in 9-H-BR. The slow phase resulting in the final proton uptake corresponds to the pK_a increase concomitant with N decay. Thus, reducing the steric interaction with the protein near position 9 of the retinal has two important consequences: (1) the time constant for reisomerization of the retinal from 13-*cis* to *all-trans* is increased 200- to 250-fold, and (2) the pK_a of the group(s) located between D96 and the aqueous phase in N is lowered from 11 to about 7.5. Whereas the first observation could be explained reasonably by an increase of the barrier to the transition state due to an entropy reduction (the reduced steric interaction enables many conformers of the chromophore in N differing in twists around single bonds (Weidlich et al., 1995)), the last finding cannot directly be linked in a straightforward manner to the reduction of the steric interaction. Apparently, it consists of an influence of the region of the 9-methyl group on residue(s) rather far away, influencing their environment in N and thereby modifying the pK_a. From this, one has to conclude that the retinal−protein interaction also influences the surface of the protein. Such an influence of an interaction in the interior of the protein on reactions at the protein surface has been described in some BR mutants (e.g., Brown et al., 1994a; Ludlam et al., 1995). Since it has been shown that with the formation of the M intermediate the cytosolic loop C-D or E-F undergoes a rearrangement (Steinhoff et al., 1994, 1995), it is conceivable that modification of the steric interaction results in the modification of this rearrangement. Further, recent experiments examining the effect of hydrostatic pressure and of modification of the cytosolic part of helix F suggest that this part of the protein undergoes movements during the rise and decay of the N intermediate, which could influence the cytosolic surface (Váró & Lanyi, 1995; Brown et al., 1995). Such a movement has also been inferred from diffraction experiments (Dencher et al., 1989; Koch et al., 1991; Nakasako et al., 1991; Subramaniam et al., 1993; Han et al., 1994; Kataoka et al., 1994), and from photoacoustic studies using thermal beam deflection (Schulenberg et al., 1994). It is interesting to note that deuterium NMR experiments indicate that the 9-methyl group changes its orientation only by 4° in the M intermediate (increase with respect to the membrane normal) (Ulrich et al., 1995; Watts et al., 1995). Thus, it could be that the interaction between this methyl group and W182 serves as pivot for the retinal movement which then induces the protein conformational changes. Delaney et al. (1995) suggested that the 13-methyl group and L93 have this function in the photocycle.

In principle, an additional intermediate could exist between N and O with a red-shifted absorption maximum but a 13-*cis* chromophore. In the previous discussion it has been tacitly assumed that in the formation of the O intermediate the reisomerization of the retinal is the rate-limiting step, which appears suggestive since the steric interaction between the chromophore and the protein has been altered and a long-lived N intermediate has been identified. From the present data, however, it cannot be excluded that the formation of this additional intermediate represents the rate-limiting step in the O formation. Since no clear evidence for this is available, this possibility will not be further discussed.

If the proton uptake process is considered also, a fourth N intermediate has to be included in addition to the three deduced from the FT-IR spectra:

$$M_2 \leftrightarrow N_1 \leftrightarrow N_2 \leftrightarrow N_3 \rightarrow N_4 \rightarrow BR$$

where the different N states are characterized in the following way: N_1: D96 deprotonated, amide-I mode at 1668 cm^{-1} not fully developed, no proton uptake; N_2: as N_1, in addition amide-I mode at 1668 cm^{-1} fully developed; N_3: as N_2, in addition D96 reprotonated; N_4: as N_3, in addition major part of proton uptake.

From the time-resolved UV−vis and FT-IR studies it is clear that the irreversible step among the N states occurs with the $N_3 \rightarrow N_4$ transition. The $N_1/N_2/N_3$ equilibrium favors N_3, but has still considerable contribution by M_2. The decay of the signal at 410 nm corresponds to the decay of this equilibrium, and thus to the $N_3 \rightarrow N_4$ transition. A careful comparison of the data obtained for WT BR at high pH (Zimányi et al., 1993) with the data presented here suggests that $N^{(-)}$ can be identified with N_2, and $N^{(0)}$ with N_4.

Removal of the 9-methyl group has a significant influence on the early part of the photocycle, explained by reduction of its steric interaction. In contrast to 9-H-BR, the photocycle of the mutant is very similar to WT BR in the corresponding time range. The efficiency of the photoreaction is normal, and at low temperature the typical K intermediate with its strong HOOP modes is formed. Similarly, the HOOP modes in L are identical to those of WT BR. Thus, the W182 → F mutation does not produce exactly the same steric effects as removal of the 9-methyl group. Apparently the lack of the methyl group somehow creates a region of reduced van der Waals contact between the retinal and the protein, allowing for greater flexibility of the chromophore even though the protein is rather rigid. Replacement of W182 by F does not create a corresponding "hole". At higher temperature or at later times, however, when the protein has been able to react to the isomerization, the two modifications have very similar consequences. This shows that at early times the interaction of the retinal close to C_9 with the protein is different from that at later times. Additional evidence for this is obtained from the data on the Trp vibrations (see below). Contrary to the early part of the photocycle for which reasonable agreement between WT BR and the mutant is obtained, drastic differences between the two systems are observed for the rise of M. It is considerably slowed in the mutant, an effect ascribed to a shifted L/M_1 equilibrium (Yamazaki et al., 1995). For 9-H-BR, however, the early phase of M rise appears even somewhat accelerated as compared to WT BR and the amplitude of M_1 is considerably larger. Therefore, the L/M_1 equilibria differ in the three systems. From the larger M_1 amplitude in 9-H-BR it can be concluded that another mechanism must explain the slow phase of M rise. If the linear reaction model is accepted, it can only be explained by a reduction in the rate constant of the $M_1 \rightarrow M_2$ transition. Thus, clear differences exist between the two modified systems. One possible interpretation for this could be an altered structural arrangement of the Schiff base and the proton acceptor D85 in L. Such altered arrangements have already been deduced for other mutants (Brown et al., 1994a). Interestingly, our data show that modification of the steric interaction influences also the important step disconnecting the Schiff base from the extracellular side and connecting it to the intracellular one.

Direct experimental evidence for the interaction of the 9-methyl group with W182 has been documented recently by the observation of a characteristic band at 3486 cm^{-1} of the L intermediate, which is missing in 9-H-BR, that was attributed to the NH stretch of W182 (Yamazaki et al., 1995). Since disappearance of this band could be caused by either an altered interaction of the chromophore with W182 or an altered interaction of the NH group of W182 with the protein, we chose a more intrinsic monitor for tryptophan perturbation. In FT-IR spectra of samples containing deuterated tryptophan or of tryptophan mutants, wagging modes around 750 cm^{-1} could be assigned in K and M spectra (Roepe et al., 1988) and those of W86 could be identified (Rothschild et al., 1989). In this work, we could unequivocally assign the corresponding mode in the L and N spectra (a similar band is also observed in the K and M spectra; Roepe et al., 1988) to W182. However, unlike the NH stretch of W182, the corresponding wagging vibration observed in the L spectrum is not influenced by removal of the 9-methyl group. It should be noted that the two vibrations characterize different parts of the indole ring. It appears reasonable that the NH stretch will be especially influenced by changes in hydrogen bonding of the NH group, whereas the wagging modes may be more sensitive to alterations in the steric interactions via van der Waals contact. Presumably, this explains the different behavior of the two modes. In the N spectrum, however, a clear effect of removal of the 9-methyl group is observed. The wagging modes of both tryptophans, i.e., W182 and W86, are missing or are very weak. This shows that the corresponding perturbations of these groups characteristic of WT BR do not take place, an observation explained by reduced steric interaction. From the three-dimensional model of bacteriorhodopsin (Henderson et al., 1990) it is not surprising that W86 is also influenced, since it is located opposite to W182, with the chromophore sandwiched in between. Although the perturbations of the two tryptophans, as deduced from the wagging modes, are very similar in L and N, the 9-methyl group influences them differently in the two states. Due to the longer times involved in the N formation in addition to the reduction of the steric constraints, the protein has now enough degrees of freedom in the neighborhood of the methyl group to accommodate the distortions caused by the isomerization of the chromophore without perturbing W86 and W182. Interestingly, in the N spectrum of the mutant W182F, we were able to detect wagging modes of the corresponding phenylalanine, which shows that now F182 is perturbed. The interaction of the 9-methyl group of the chromophore with F182 is evidently sufficient to retain the perturbation of W86 observed for WT BR. Since the modes due to F182 are only observed in the N spectrum, it is not surprising that this is the state most drastically influenced by the mutation.

Prolonged lifetimes of the N intermediate have been described for other modifications. The question arises whether the similarity observed for 9-H-BR and the W182F mutant is accidental. However, there is very specific agreement in a multitude of observed alterations—fast M decay, four N intermediates, long lifetime of N, similar kinetics for proton uptake on the cytosolic surface—that point to a common molecular cause. The FT-IR spectra and the time courses of proton uptake indicate that proton transfer from D96 to the Schiff base is facilitated in the two systems. Therefore, the question arises which are the disadvantages

one has to trade in. Apparently, the advantage of fast proton transfer is counterbalanced by a disadvantageous pK_a reduction of the external groups and, most importantly, by the slow reisomerization of the chromophore, that limits the number of protons transported at high light intensities.

In this paper we have substantiated our earlier conclusion that steric interactions between the chromophore and the protein play an important role in optimizing the photocycle of bacteriorhodopsin. We have emphasized especially the importance of the region around C_9 of the chromophore interacting with W182. Perturbation of this interaction, by changing either the chromophore (9-H-BR) or the protein (W182F), has very similar effects on the later part of the photocycle (N rise and decay). Since the two modifications of the interaction are not identical, the earlier part of the photocycle is differently influenced. An important result is the observation that the reduced steric interaction influences in the N state of both systems the pK_a of the proton acceptor/donor group(s) located between D96 and the aqueous phase close to the surface of the protein. Interestingly, for rhodopsin the retinal−protein interaction near the 9-methyl group is also of importance (Ganter et al., 1989). Removing this interaction abolishes the conformational changes of the cytosolic loops detected recently in the metarhodopsin II state (Ganter et al., 1992; Farahbakhsh et al., 1993; Resek et al., 1993) which are thought to be of importance for the interaction of activated rhodopsin with transducin (König et al., 1989; Franke et al., 1990).

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