**Static and Time-Resolved Step-Scan Fourier Transform Infrared Investigations of the Photoreaction of Halorhodopsin from *Natronobacterium Pharaonis*: Consequences for Models of the Anion Translocation Mechanism**

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**ABSTRACT** The molecular changes during the photoreaction of halorhodopsin from *Natronobacterium pharaonis* have been monitored by low-temperature static and by time-resolved step-scan Fourier transform infrared difference spectroscopy. In the low-temperature L spectrum anions only influence a band around 1650 cm⁻¹, tentatively assigned to the C=N stretch of the protonated Schiff base of L. The analysis of the time-resolved spectra allows to identify the four states: K, L₁, L₂, and O. Between L₁ and L₂, only the apoprotein undergoes alterations. The O state is characterized by an all-trans chromophore and by rather large amide I spectral changes. Because in our analysis the intermediate containing O is in equilibrium with a state indistinguishable from L₂, we are unable to identify an N-like state. At very high chloride concentrations (>5 M), we observe a branching of the photocycle from L₂ directly back to the dark state, and we provide evidence for direct back-isomerization from L₂. This branching leads to the reported reduction of transport activity at such high chloride concentrations. We interpret the L₁ to L₂ transition as an accessibility change of the anion from the extracellular to the cytosolic side, and the large amide I bands in O as an indication for opening of the cytosolic channel from the Schiff base toward the cytosolic surface and/or as indication for changes of the binding constant of the release site.

**INTRODUCTION**

In contrast to the light-driven proton pump bacteriorhodopsin, for which many details of the proton transfer and energy conversion mechanism are understood at a molecular level (Brown et al., 1998; Haupts et al., 1997), much less is known about the corresponding aspects of the related chloride pump halorhodopsin (Oesterhelt, 1995). Although quite a number of halorhodopsins have been identified (Mukohata et al., 1999), only those from *Halobacterium salinarum* (sHR) and *Natronobacterium pharaonis* (pHR) have been extensively studied. Bacteriorhodopsin and halorhodopsins show a surprising homology between their primary structures (Lanyi et al., 1990) with the exception of the key residues D85 and D96, which function in bacteriorhodopsin as proton acceptors and proton donors for de- and reprotonation of the Schiff base. In pHr, the corresponding sites are occupied by T126 (111) and A137 (122), respectively (the corresponding numbers of sHR are placed between parentheses). As expected from these replacements, the normal photocycle of halorhodopsins does not contain an intermediate with a deprotonated Schiff base (M-like state).

In a recently published structure of sHR, chloride in its binding site could be identified. It accepts hydrogen bonds from two water molecules and from a serine side chain. The chloride is close to the Schiff base, although in unfavorable position for hydrogen bonding (Kolbe et al., 2000). Together with data on the photocycle and studies of site-directed mutants a detailed model on the chloride transport mechanism has been developed. In addition, time-resolved electric measurements have correlated chloride transport steps with formation of intermediates (Kalaidzidis et al., 1998; Ludmann et al., 2000).

In previous investigations, the photocycles of sHR and pHr have been studied by time-resolved ultraviolet-visible (UV-vis) spectroscopy (Kalaidzidis et al., 1998; Ludmann et al., 2000; Scharf and Engelhard, 1994; Váró et al., 1995a,b,c); the latter results on pHr being in variance with the data on sHR. It appears that the main difference between the two pigments is the lack of accumulation of the O state in the latter system, probably because of kinetic reasons (Váró et al., 1995c). The capability of light adaptation represents another difference between the two systems, sHR containing ~50% all-trans retinal in the dark and 75% in white light, whereas pHr contains 85%, irrespective of the illumination condition (Váró et al., 1995a; Zimányi and Lanyi, 1997).

Information on molecular changes during the photocycle has been obtained by infrared difference spectroscopy (Bouschê et al., 1991; Braiman et al., 1994; Rothschild et al., 1988; Rüdiger et al., 1995; Walter and Braiman, 1994) and by resonance Raman spectroscopy.
explain the inhibition of chloride transport at such extreme conditions. The results are discussed within the framework of possible pumping mechanisms.

MATERIALS AND METHODS

Preparation of halorhodopsin samples

Halorhodopsin from *Natronobacterium pharaonis* was isolated according to Scharf and Engelhard (Scharf and Engelhard, 1994). Halorhodopsin expressed in *Escherichia coli* was prepared using the method of Hohenfeld et al. (1999). Reconstitution of pHR into purple membrane lipids was performed following the procedure described in Losi et al. (Losi et al., 1999).

Preparation of samples for infrared spectroscopy

Approximately 2 nmol of halorhodopsin reconstituted into lipids from *Halobacterium salinarum* was suspended in 100 µl buffer, 10 mM Tris/H$_2$SO$_4$ (pH 7), deposited onto a BaF$_2$ window, dried under nitrogen, and rehydrated with ~0.5 µl H$_2$O. Subsequently, the sample was sealed with a second window. Thus, the samples contained 1 µMol sulfate at an approximate concentration of 2 M. Depending on the experiment, the respective amount of salt was added in order to obtain the required anion concentration. It should be noted that the sample volume is very approximate. Therefore, the salt and buffer concentrations in the infrared samples may vary by ± 50%.

Time-resolved infrared measurements

Time-resolved step-scan FTIR measurements with 30-ns time resolution have been performed essentially as described (Rödig et al., 1999), with the exception that the fast (30-ns time resolution) and the slow (40-ns time resolution) measurements have been performed separately. With the fast setup, an ac-coupled amplifier has been used, limiting the covered time range to 100 µs. For sample excitation, a frequency-doubled Nd:YAG laser was used delivering 1.5 to 2 mJ to the sample with an approximate area of 1 cm$^2$. Spectral resolution is 8 cm$^{-1}$, eight signals have been averaged per interferogram sampling point, and four measurements are averaged. Measurements have been performed at room temperature. Before the infrared measurements, the same samples have been tested with respect to published data in a simple set-up for time-resolved UV-vis measurements.

Static low-temperature FTIR measurements

Static infrared difference spectra at 123 and 193 K have been obtained with a home-built cryostat inserted into a Bruker IFS 113v FTIR instrument, equipped with an MCT detector, essentially as described previously (Gervert and Siebert, 1986). Spectral resolution is 2 cm$^{-1}$, and ~500 scans have been averaged before and after the illumination. For illumination, a slide projector connected to fiber optics has been used. To obtain the K state, the sample was illuminated with wavelengths between 490 and 550 nm at 123 K. To obtain the L state, the sample has been illuminated at 193 K with wavelengths longer than 610 nm.

Data evaluation of time-resolved infrared spectra

The time courses of the time-resolved spectra have been analyzed with the global fit program (MEXFIT)(Müller and Plessier, 1991) as described previously (Rödig et al., 1999). The resulting amplitude spectra have been converted into intermediate spectra, using the unidirectional reaction model without back-reactions (Chizhov et al., 1996), which has been successfully applied to the analysis of time-resolved infrared spectra of bacteriorhodopsin.
sin (Rödig et al., 1999). With this method, the derived intermediates generally are mixtures of pure states, the mixtures being in a fast equilibrium. As stated before, this kind of analysis provides a bias-free method of interpretation of time-resolved spectra, which does not suffer from the problem of free parameters. On the other hand, the intermediate spectra can be better described in molecular terms than the amplitude spectra because the latter are strongly influenced by the kinetic constants. We do not claim, however, that this reaction model represents the true path of the photocycle. In the analysis, there is generally the freedom for the sequence of the time constants. However, it turned out that only the series with increasing time constants provides reasonable and consistent intermediate spectra. In the following, we differentiate between intermediates, possibly being mixtures, and states representing pure states.

RESULTS

Low-temperature FTIR difference spectra

In Fig. 1, the low-temperature FTIR difference spectra of pHR in 500 mM NaCl are shown for comparison with the time-resolved spectra that are presented below. Although the spectra are very similar to earlier published data of sHR (Bousché et al., 1991; Rothschild et al., 1988) which reflects the homology of the two systems, a few remarks should be pointed out. At 123 K, the red-shifted K intermediate should be stabilized. However, no clear evidence for the ethylenic mode of the chromophore of this intermediate can be identified (assignments of chromophore bands are made on the basis of the corresponding bands of bacteriorhodopsin) (Fodor et al., 1988; Gerwert and Siebert, 1986; Smith et al., 1985). A band at 1514 cm\(^{-1}\), detected in the corresponding spectrum of sHR (Rothschild et al., 1988), is not present in pHR. The band at 1500 cm\(^{-1}\) has a position too low for this assignment. For the correlation between the visible absorption maximum and the position of the ethylenic stretching mode of protonated retinylidene Schiff bases, see Heyde et al. (1971) and Ottolenghi (1980). It is striking that, in comparison to the spectrum obtained at 193 K, the band at 1525 cm\(^{-1}\), mainly caused by the ethylenic mode of the dark state, has much lower intensity, indicating considerable spectral overlap between the bands of the dark state and of the 123 K photoproduct (the negative band at 1006 cm\(^{-1}\), probably representing the methyl wagging mode of the chromophore, is used for normalization). Therefore, one would also expect considerable overlap between the corresponding visible absorption spectra (see discussion of the time-resolved data) which indeed was found (Chizhov and Engelhard, submitted).

Congruent with published data for K of sHR (Rothschild et al., 1988), the positive band at 1536 cm\(^{-1}\) is interpreted as amide II spectral changes, and the band at 967 cm\(^{-1}\), having strong intensity, is assigned to a hydrogen-out-of-plane mode of the chromophore. The strong intensity indicates a highly twisted chromophore (Fahmy et al., 1989, 1991). In contrast to the K spectrum of bacteriorhodopsin (Siebert and Mäntele, 1983), the fingerprint bands of the dark state between 1260 and 1140 cm\(^{-1}\) exhibit considerable fine structure that cannot be explained by different chromophore modes. They represent C–C stretching vibrations coupled to CH bending modes of the chromophore, and, thus, they probably indicate a structural heterogeneous chromophore in the dark state at low temperature. Interestingly, such a heterogeneous chromophore distribution, which is redistributed by illumination and reduced or even abolished at higher temperatures, has also been described for sHR from low-temperature UV-vis spectroscopy (Zimán-yi et al., 1989). Similarly, this temperature effect is also observed in the spectrum taken at 193 K (Fig. 1, lower trace), which displays a considerably reduced fine structure in the fingerprint region of the dark state. It vanishes completely at room temperature (see below). Therefore, it cannot be caused by a possible excitation of the 13-cis,15-syn chromophore. The 193-K spectrum can be assigned to the low-temperature L\(_1\) state, because it differs from the spectrum obtained at 250 K (Bousché et al., 1991), which has been ascribed to L\(_2\), as reported previously (Chon et al., 1999).

Anions have been shown to influence the bands at 1690 and 1631 cm\(^{-1}\) in sHR (Walter and Braiman, 1994). Therefore, we have repeated the measurements of the L\(_1\) state in the presence of 500 mM NaI and 500 mM KBr. A careful analysis with 2 cm\(^{-1}\) spectral resolution and zero-filling of

![FIGURE 1](image-url)
4 did not reveal differences larger than 0.4 cm\(^{-1}\) for these two bands, the lower limit being mainly imposed by variations observed among spectra of the same kind. Obviously, these bands seem to be less sensitive in pHR than in sHR-(Braiman et al., 1994; Chon et al., 1999). The 1690 cm\(^{-1}\) vibrational mode has been ascribed to a guanidinium mode, tentatively assigned to R123(108) (Braiman et al., 1994; Rüdiger et al., 1995). This interpretation, however, has been questioned because \(^2\)H\(_2\)O does not evoke the expected isotope shift (Chon et al., 1999), which is in line with our own observations (not shown).

The other band at 1631 cm\(^{-1}\) is located in the region characteristic for the C= N stretching band of the protonated Schiff base in the dark state for both, sHR (Alshuth et al., 1985; Maeda et al., 1985; Pande et al., 1989) and pHR (Gerscher et al., 1997). Also in this case, the anion influence is controversial. Whereas for the C= N stretch in sHR, an anion dependence was described (Maeda et al., 1985; Pande et al., 1989; Walter and Braiman, 1994), which has not been seen in pHR (Gerscher et al., 1997). One has to keep in mind that in the infrared studies the observed anion effects around 1690 and 1631 cm\(^{-1}\) may be due to changes of amide I modes that occur in sHR but not in pHR. The C= N stretch of K may be located at 1620 cm\(^{-1}\), and that of L at 1650 cm\(^{-1}\), the latter being approximately in agreement with resonance Raman data. We have found that in L this band is weakly anion dependent (Cl\(^-\): 1650.0 cm\(^{-1}\), Br\(^-\): 1649.1 cm\(^{-1}\), I\(^-\): 1648.5 cm\(^{-1}\), data not shown) in approximate accordance with the resonance Raman results. However, due to the overlap with strong amide I bands, the C= N stretch can only be unequivocally assigned if labeled retinals are used.

**Time-resolved infrared difference spectra (30-ns time resolution and 500 mM NaCl)**

The 100-ns spectrum obtained with 30-ns time resolution (Fig. 2) resembles the static K spectrum obtained at 123 K (Fig. 1). The large intensity of the hydrogen-out-of-plane (HOOP) mode at 970 cm\(^{-1}\) shows that it is not a property of the low-temperature state, although the position is slightly shifted to higher wavenumbers (all features below 950 cm\(^{-1}\) are not further discussed because this spectral range is severely distorted by noise). The bands at 1550 cm\(^{-1}\) (negative) and 1536 cm\(^{-1}\) (positive) are further common features, although the intensity of the former is much stronger in the time-resolved spectrum. The reduced fine structure of the bands in the finger print region (1140 to 1260 cm\(^{-1}\)), as compared to those of the low temperature K spectrum, can only partly be explained by the lower spectral resolutions (8 cm\(^{-1}\) versus 2 cm\(^{-1}\)). As discussed above, the heterogeneity is reduced and finally abolished at higher temperatures. Despite the lower resolution, the two positive bands at 1198 and 1188 cm\(^{-1}\) should have been observed in the time-resolved studies. It is interesting to note that the K spectrum of bacteriorhodopsin, in which at low temperature a similar splitting of the positive band is observed, although with different amplitudes, displays a similar sensitivity toward temperature (Siebert and Mändele, 1983; Weidlich and Siebert, 1993). Apparently, the low temperature imposes slightly different chromophore geometries in the K states of BR as well as of pHR, a conclusion that can also be deduced from the changes of the HOOP modes in both systems. The amide I bands are more intense in the time-resolved than in the static spectrum, indicating larger structural changes. The difference band around 1740 cm\(^{-1}\) caused by protonated D156 (141) is less affected.

The analysis of the kinetics obtained with 30-ns time resolution yields only a single half-time of 400 ns, in reasonable agreement with UV-vis data (Váró et al., 1995a) (Chizhov and Engelhard, submitted). Obviously, in this shorter time range the 13-cis,15-syn retinal conformer in pHR does not contribute to the absorbance changes. Time-resolved step-scan FTIR investigations of sHR have been recently published (Dioumaev and Braiman, 1997). Although many spectral features agree, the reported long life-
time for the K intermediate (360 μs) is clearly in conflict not only with the present data but also with time-resolved UV-vis data of sHR (Váró et al., 1995a).

**Time-resolved infrared difference spectra (400-ns time resolution and 500 mM NaCl)**

Representative spectra obtained with 400-ns time resolution for times longer than 100 ns are shown in Fig. 2. The complete set of spectra has been analyzed according to the procedure described in Materials and Methods. The global fit reveals four time constants. The corresponding amplitude spectra are presented in Fig. 3. The first time constant of 1.3 μs is considerably affected by the time resolution of the experimental set-up, which only allows to evaluate the data beginning at 1.5 μs. From the amplitude spectra, the spectra of the intermediates have been derived as described in Materials and Methods (Fig. 4). The first spectrum with the half-time of 1.3 μs is very similar to the 100-ns spectrum of K shown in Fig. 2. However, due to the limited time resolution, it already contains some admixture of L as revealed by the reduced intensities of the HOOP mode at 970 cm⁻¹ and of the negative band at 1550 cm⁻¹. The spectrum of the next intermediate (decay time of 57 μs) is essentially identical to the spectrum obtained at 10 μs after the flash (Fig. 2). Contributions from the K state are not present any longer, as is evident from the missing HOOP mode. Thus, this spectrum corresponds to a pure L state. This observation is in contrast to the finding of Váró et al. (1995a), who described the presence of ~30% K during the lifetime of the L state. The amplitude spectrum of Fig. 3 (denoted with 1.3 μs) reflects, due to the large separation of the two first time constants, the transition from K to L.

The amplitude spectrum (Fig. 3) with half-time of 57 μs has only bands between 1650 and 1500 cm⁻¹ that are very broad. The absence of bands assigned to fingerprint modes excludes that the bands at 1512 and 1557 cm⁻¹ originate from ethylenic stretch modes of the chromophore. Instead, all the bands must be assigned to amide I (1612 and 1651 cm⁻¹) and amide II (1557 cm⁻¹ and 1512 cm⁻¹) modes. Thus, this transition solely involves changes of the protein and represents, with respect to UV-vis data, a spectrally silent transition.

The resulting intermediate spectrum (half-time, 1.1 ms) is very similar to the previous spectrum. Therefore, the corresponding two species are defined as L₁ (half-time, 57 μs) and
L₂ (half-time, 1.1 ms). In addition to the amide changes discussed, a difference band at 1740 cm⁻¹ reappears, although with low amplitude. It is at same position as observed in the K spectra, and is therefore assigned to D156(141). Two L intermediates have also been detected in the photoreaction of sHR using static FTIR difference spectroscopy (Chon et al., 1999). Because of the different species and the different technique, the spectra are difficult to compare.

The following amplitude spectrum (half-time, 2.7 ms) is characterized by intense chromophore as well as protein bands. The large vibrational modes between 1700 and 1600 cm⁻¹ mainly reflect changes of amide I bands. The negative band at 1634 cm⁻¹ may be assigned to the C=N stretch of the protonated Schiff base of this new intermediate. If so, the positive band at 1652 cm⁻¹ would then contain contribution from the C=N stretch of L₂. However, due to the overlap with amide modes, an unequivocal assignment can only be made with isotopically labeled chromophores. The bands in the fingerprint region indicate that the chromophore undergoes alterations. In this time range the rise of the O intermediate, which absorbs above 600 nm, has been reported ((Váró et al., 1995a; Chizhov and Engelhard, submitted; our own control measurements). In agreement with this, the negative band at 1512 cm⁻¹ indicates the rise of the ethylenic mode of the red-shifted photoproduct. All the features discussed can also been seen in the intermediate spectrum. The reduced intensity of the fingerprint mode of the dark state at 1169 cm⁻¹ (C₁₀–C₁₁ stretch) indicates that the chromophore is partially isomerized to the all-trans geometry. However, from the comparison with the corresponding spectrum of O of bacteriorhodopsin, a positive band would have been expected around this position (Hessling et al., 1993; Zscherp and Heberle, 1997). Furthermore, the small intensity of the ethylenic mode at 1512 cm⁻¹ shows that this intermediate cannot be due only to a red-shifted intermediate, but that it must rather represent a mixture (see below). From the broad feature around 970 cm⁻¹ representing several HOOP modes one has to conclude that the chromophore is in a twisted conformation. Whereas all the previous intermediate spectra represent rather pure states, correctly described by the reaction model, this last intermediate spectrum containing a mixture of states is a clear consequence of the adopted linear reaction scheme without back-reactions. The described spectral changes over the complete time range can be nicely followed in the time traces at selected wavenumbers shown in Fig. 5, in which the traces of the global fit are also presented. Due to memory limitations of the transient recorder board, we were unable to follow the photocycle in one single measurement beyond the time range shown. Therefore, we were unable to detect the HR' → HR reaction with a half time of ~20 ms (Váró et al., 1995a). However, the pronounced decay of all the traces before 10 ms clearly demonstrates that HR’ cannot be very different from HR (the dark state of pHR).

Because the first three half times are well separated, the first three intermediate spectra are very similar to the first three time-resolved spectra taken at times before the respective decay has substantially started (Fig. 2).

**Time resolved measurements of chloride depleted pHR**

In the case of pHR a blue species (pHRblue) with an absorption maximum at 600 nm can be generated by suspending it in distilled water. The original purple state can be regained by the addition of monovalent anions (Scharf and Engelhard, 1994). The red-shift is interpreted by the removal of the counterion from the protonated Schiff base. Surprisingly, the investigation of the photocycle of pHRblue in the infrared was impossible, because the necessary reduction of water for the preparation of the hydrated film samples led to a reconversion of the blue form back into the purple form. The effect is neither caused by residual anions present if an elemental analysis and the binding constant of ~3 mM (Scharf and Engelhard, 1994) are taken into account. Nor can the transition be due to anions dissolved from the infrared window (BaF₂), because the same observation has been made for films on ZnSe or polyethylene support. The blue to purple conversion had already occurred at a quite high water content of the sample, which would not yet allow...
infrared measurements. This is different from bacteriorhopdopsin, in which the transition occurs at very low water content (Fahmy and Siebert, 1990).

Despite the lack of transportable anions, our control measurements in the UV-vis showed that the photocycle of this anion-depleted hydrated film sample was identical to that in the presence of 10 mM chloride, but not to that of pH7 blue in solution. Furthermore, time-resolved infrared measurements at 10 mM NaCl revealed no differences. In the following, the time-resolved infrared spectra of such a hydrated film sample without chloride (denoted “measurement without chloride”) are discussed.

Within the signal/noise ratio, the K spectrum obtained with 30-ns time resolution is undistinguishable from the spectrum obtained with 500 mM chloride (not shown). The analysis of the data collected with 400-ns time resolution yielded half-times of 1.6, 48, 1723, and 6249 µs. With the exception of the latter, they compare well with those of the 500-mM measurement. Also, the corresponding amplitude and intermediate spectra reveal no differences within the noise level, and they are, therefore, not reproduced here. The small influence of anions on the band around 1650 cm$^{-1}$ observed at low temperature would not have been detectable in these data. It is important to emphasize that the same spectral features are observed in the $L_1 \rightarrow L_2$ transition, i.e., this transition does not depend on the presence of chloride. However, the amplitude spectrum with half-time of 1.7 ms and the corresponding intermediate spectrum with half time of 6.2 ms are clearly different (Fig. 6). In the amplitude spectrum the larger ethylenic mode at 1511 cm$^{-1}$ indicates that a higher fraction of O is produced. Its real position has to be up-shifted by 3 cm$^{-1}$ due to the overlap with the band of the dark state, and, thus, an absorption maximum between 600 and 610 nm can be deduced using a ratio $\Delta \lambda_{\text{max}} / \Delta \nu$ of $\sim 3.5$ (Ottolenghi, 1980) and the corresponding values of O of bacteriorhopdopsin [$\Delta \lambda_{\text{max}}$ 630 nm (Chizhov et al., 1996); C = C stretch: 1509 cm$^{-1}$ (Smith et al., 1983)]. The large amplitude of bands in the amide I region indicate considerable changes in the peptide backbone. Both features are equally evident in the intermediate spectrum (compare with 1.1 and 2.7 ms spectra in Fig. 4). The two positive bands in the fingerprint region at 1196 and 1165 cm$^{-1}$ (Fig. 6) clearly demonstrate the all-trans geometry of the chromophore in O (Zscherp and Heberle, 1997). The all-trans geometry has also been deduced from static FTIR investigations using steady-state illumination at low and high chloride concentration (Váró et al., 1995a). Because of the lower noise in our data, the spectra are difficult to compare. The broad HOOP mode around 964 cm$^{-1}$ and the possible shift of the C=N stretch from 1655 to 1632 cm$^{-1}$ have already been mentioned in the presentation of the last spectrum of Fig. 4. These bands now have larger amplitudes due to the higher content of O. Indeed, in the intermediate spectrum, the negative band at 1632 cm$^{-1}$ is not present, indicating the total compensation of the band due to the C=N stretch of the dark state by another band of the O intermediate, supporting its identification with the C=N stretch of O.

Based on the analysis of time-resolved UV-vis data of pH7 at different NaCl concentrations (Chizhov and Engelhard, submitted), the intermediate spectrum (6.2 ms) should essentially represent a pure O state. Therefore it can be used to decompose the 2.7 ms intermediate spectrum of Fig. 4 which does not reflect that of a pure single component. A fraction of the 6.2-ms spectrum (Fig. 6) is subtracted from the 2.7-ms spectrum (Fig. 4), compensating the characteristic ethylenic stretch and HOOP modes of O (dashed line in Fig. 4, multiplied by 2.62). The comparison reveals, within the noise limits, no differences from the 1.1-ms spectrum. Obviously, our data show that O is in equilibrium with $L_2$. An N-like state, as it was described for bacteriorhopdopsin could not be identified. The factor of 2.62 (see legend to Fig. 4) indicates that $\sim 62%$ O are present in the mixture.

**Time resolved measurements at high chloride concentration**

In order to obtain more information on the chloride transport process, we performed time-resolved infrared measurements at very high chloride concentrations (6 M), at which the chloride release process may be impaired (Bamberg et al., 1984). At high salt concentrations, the infrared transmission of the samples is very poor, resulting in much larger noise in the spectra. The kinetic analysis of the measurements with 400-ns time resolution revealed half-times of 0.4, 61, 955, and 4900 µs (Fig. 7, amplitude spectra (Fig. 8), intermediate spectra). Because of the large noise, we omit the K spectrum. The 61- and 955-µs intermediate spectra very closely resemble those of L1 and L2, respectively. Also...
the half-times are not drastically changed, with L2 decaying somewhat faster at very high salt concentration. However, whereas in the L1 → L2 transition at 500 mM chloride or without chloride only protein modes are involved, small contributions of the chromophore can be clearly seen in the fingerprint region at 6 M chloride. Aside from this feature, the corresponding amplitude spectrum, denoted as 61 s, essentially represents the difference between L1 and L2 discussed previously. The bands in the fingerprint region very closely resembles the pattern observed in the L2 → L2/O transition depicted in Fig. 3 (1.1-ms trace). It can be concluded that in the L2 state at 6 M chloride a small percentage of the chromophore is already back-isomerized to all-trans, and that the chromophore geometry in this all-trans species must be similar to that of O, i.e., different from that of the dark state. Under these conditions, the O-intermediate itself is not formed because an absorbance increase at 1512 cm⁻¹ is absent. Surprisingly, the 955-µs amplitude spectrum represents to a large extent already the decay to the initial state, because it contains most of the negative bands of the intermediate spectra representing the dark state. Consequently, the last intermediate spectrum has a considerable reduced amplitude, but is otherwise very similar to the spectrum of L2. However, the bands in the amide I region, if normalized to the ethylenic mode at 1524 cm⁻¹, have considerably larger amplitude. Superficially, they resemble the bands observed in O, but a careful comparison reveals clear differences in band position. We call this state L2'.

**DISCUSSION**

**Characterization of the intermediates**

One difficulty in the analysis of the photoreaction of pHr could arise from the partial presence of the 13-cis,15-syn geometry of the chromophore (10 to 15%) in the dark, as well as in the light-adapted state (Váró et al., 1995a). Flash photolysis experiments comparing sHR and pHR have shown that this species does not contribute to absorbance changes at times longer than 100 ns (Váró et al., 1995a). Therefore, we can also neglect this species in our time-resolved infrared measurements. This is corroborated by the analysis of the data with higher time-resolution, which reveals only one time-constant reflecting the decay to L. Thus, it appears adequate to also ignore this species in the low-temperature spectra.

The low temperature L spectra provide important information on the interaction of anions (chloride, bromide,
iodide) with the protein: 1) the band structure around 1690 cm\(^{-1}\) is not influenced by anions in contrast to sHR; 2) no influence of anions can be seen around 1630 cm\(^{-1}\) in contrast to sHR, i.e., the C=N stretch of the Schiff base of the dark state is not affected; 3) we tentatively confirm the effect of anions on the C=N stretch of the L state (Gerscher et al., 1997). In principle, the size of the anions should have an influence on the C=N stretching vibration if the binding site is close to the Schiff base. Although the ionic radii of anions like iodide without affecting the direct Schiff base environment.

Because \(^{2}\)H\(_2\)O causes no change in the band pattern around 1690 cm\(^{-1}\), we agree with earlier conclusions (Chon et al., 1999) that it cannot be caused by R123(108); pHR and sHR show a very similar band pattern, indicating that it has similar molecular origin. This interpretation is in line with the published structure, which indicates only an indirect interaction of the anion with R123(108) via two water molecules. It is not clear which molecular group causes this pattern. However, the lack of anion influence indicates, as compared to sHR, a reduced interaction of the anions with the protein. This observation further corroborates a less restricted binding site. This may explain why pHR, in contrast to sHR, pumps nitrate as effectively as chloride, and, probably, to a small extent, sulfate (Duschl et al., 1990). These differences between pHR and sHR might also be the cause for the observation that pHR, but not sHR, forms a blue membrane.

In the amplitude spectrum reflecting the transition of K to L (1.3 \(\mu s\), one would expect to observe the ethylenic modes of the chromophore. The C=C stretch of the L-intermediate, having its maximum at 520 nm (Ludmann et al., 2000; Váro et al., 1995a; Chizhov and Engelhard, submitted), could correspond to the band 1552 cm\(^{-1}\) and that of K to the low-frequency part of the broad band at 1534 cm\(^{-1}\) if, as suggested in Results, an absorption maximum of K close to that of the dark state is assumed. The lack of a positive band at 1512 cm\(^{-1}\) excludes an absorption maximum of K close to 600 nm. Thus, this amplitude spectrum also supports an absorption maximum of K around 575 nm, in agreement with Duschl et al. (1990) and Chizhov and Engelhard (submitted).

As expected, the main differences between the spectra of time-resolved L\(_1\) and low-temperature L\(_2\) are caused by the different spectral resolutions and by the fine structure discussed above. Most of the broadened bands can be reproduced by convoluting the bands of the low-temperature spectrum with a broader bands. However, the low intensity of the two bands at 1539 and 1553 cm\(^{-1}\), using the bands between 1650 and 1630 cm\(^{-1}\) for normalization, cannot be explained in this way. In resonance Raman experiments the C=C stretching mode of L in the presence of chloride (denoted as pHR\(_{520}\)) has been located at 1550 cm\(^{-1}\) (Gerscher et al., 1997). Because in the infrared amide II modes may also contribute, we can conclude that the time-resolved L state is characterized by an ethylenic stretching mode with very low infrared intensity, similar to the L state of BR (Rödig et al., 1999). Furthermore, the composite character of the band at 1533 cm\(^{-1}\) does not allow to identify possible anion-induced shifts as have been described in the resonance Raman experiments. The band at 1536 cm\(^{-1}\) also has considerably higher intensity in the low-temperature spectrum and is attributed to an amide II mode. The isomerization of the chromophore in the more rigid protein structure at low temperature probably causes locally larger distortions in the peptide backbone. Thus, at room temperature, the early part of the photocycle exhibits considerably more dynamic behavior than at low temperature: protein changes are larger, but smaller in L.

Measurements in the absence of chloride (or at 10 mM NaCl) allowed us to obtain an essentially pure O spectrum (Fig. 6), an intermediate whose existence has also been proposed from time-resolved UV-vis investigations (Váró et al., 1995a). The spectral properties of this red-shifted intermediate has been explained by canceling the interaction of the Schiff base with the anion. The twisted chromophore geometry deduced from the HOOP modes appears to be a property of intermediates formed after thermal back-isomerization, since similar HOOP modes have also been reported for O of bacteriorhodopsin (Rödig et al., 1999; Smith et al., 1983) and that of the Y185F bacteriorhodopsin mutant (He et al., 1993). Our data clearly shows that the chromophore geometry in this intermediate is all-trans. Thus, one could speculate that this intermediate is similar to blue pHR obtained by removal of the anion from its extracellular binding site (Scharf and Engelhard, 1994). However, the strong HOOP modes in O, indicating a non-relaxed chromophore, argue against this identity. Attenuated total reflection (ATR)-FTIR titration experiments, which can be performed at higher water content, are in progress to characterize the dark state in the absence of a monovalent anion.

The rise of the pure O state under anion-free conditions is well separated from its precursor L\(_2\). Raising the chloride concentration to 500 mM chloride establishes a fast equilibrium between O and L\(_2\) with a transient concentration of \(\sim 60\%\) O (Fig. 4). It is important to emphasize that the spectral properties of this L\(_2\) state are, within the noise level, indistinguishable from the L\(_2\) intermediate determined as a pure state in the time-resolved infrared difference spectra. This observation is in contrast to time-resolved UV-vis studies, which indicate the presence of an N-like species with an, with respect to L, red-shifted absorption maximum
(Váró et al., 1995a). The question arises if these discrepancies concerning the existence of an N intermediate are due to the evaluation method. Generally, a reaction model in which an extra component is introduced would require an additional time constant. However, within the noise level of our data, an additional time constant does not reduce the error in the global fit and does not improve the sum of residuals. This shows that, if indeed an N state is present, its spectral properties must be very similar to those of the L2 state, which is in contrast to the above cited UV-vis analysis. Recent time-resolved UV-vis investigations show the presence of an N state that, however, has an absorption maximum indistinguishable from that of L (Chizhov and Engelhard, submitted). The identification of transitions that are spectrally silent also in the infrared requires very high signal/noise ratio. Nevertheless, although there are some deviations in details of the reaction models, all assume that the O state, which has an all-trans chromophore, is in equilibrium with a species with a 13-cis isomer.

At present we can only speculate why the anion-free form of the hydrated film samples displays spectral properties and a photocycle identical to that of pH7 at 10 mM NaCl. Although the anion-free form of sHR in solution displays an absorption maximum at 570 nm, i.e., even little blue-shifted from the chloride-bound form, the photocycle is completely different (Oesterhelt, 1995). Therefore, the two systems cannot be compared. The absorption maximum in the chloride-free infrared sample around 578 nm requires the presence of a counterion, substituting for the chloride. Sulfate, which is present in our sample (originating from the Tris/H2SO4 buffer), cannot be involved because the same transformation from the blue to the purple also occurs if distilled water is used. Also, a simple concentration increase of any negatively charged species seems unlikely, because otherwise it should have also been possible to induce the purple form in suspensions of pH7 without a transportable anion. The possibility that a residue of halorhodopsin itself takes over the function of the counterion (e.g., D252(238) by movement of R123(108) toward the extracellular side) can be excluded because the photocycle should be considerably different from that involving an active anion transport. A likely explanation could be that the close neighborhood of adjacent membranes in the hydrated films induces alterations in the anion binding site, such that an OH− ion substitutes for chloride even at pH 7. Further experiments are required to explain the absorption maximum and the photocycle kinetics of the chloride-free hydrated film samples. However, irrespective of the mechanism, the time-resolved infrared studies of the chloride-free samples confirm that the anions have very little influence on the spectra.

At 6 M NaCl, the photocycle is drastically changed. 1) With the formation of L2 the chromophore is already partially backisomerized to all-trans, the corresponding chromophore geometry being similar to that of O but different from that of the dark state. 2) An intermediate is formed that is different both from this L2 and different from O, and because of its greater similarity to L2 we have termed it L2′. A further peculiarity of this intermediate is the small amplitude of negative chromophore bands, demonstrating that a considerable fraction of L2 has already decayed to the initial state with the formation of L2′. If the unidirectional reaction scheme would be applied for this transition, this would show that L2′ is in equilibrium with the dark state. Because this putative equilibrium is completely transformed into the dark state with the last step, i.e., irreversibly, we regard this interpretation as unlikely. Of course, we cannot exclude that the putative equilibrium does not contain the dark state, but a state very similar to that, and that the irreversible transition is from this pseudo-dark state to the dark state. However, it is difficult to reconcile an irreversible step between two species differing very little in their molecular properties as determined by infrared spectroscopy.

As an alternative, we propose a branching of the reaction cycle at the L2 state, according to the following scheme:

\[
\text{L2} \xrightarrow{k_1} \text{L2}′ \xleftarrow{k_2} \text{HR}
\]

Neglecting the fast formation of L2, the kinetic analysis shows that two rate constants, k1 and k2, describe this scheme, whereby \( k_1 = k_{\text{LL}} + k_{\text{LHR}} \) and \( k_2 = k_{\text{LHR}}(k_{\text{LL}}', k_{\text{LHR}}, \) and \( k_{\text{LHR}} \) being the microscopic rate constants for the decay of L2 to L2′, L2 to HR, and of L2′ to HR, respectively. Thus, the amplitude spectrum with the half-time of 4.9 ms describes the decay of the L2′ as assumed (Figs. 7 and 8), whereas the amplitude spectrum with half-time of 955 μs contains contributions of the intermediate L2 and L2′. Using the fact that the rise of L2 is much faster than its decay, the intermediate spectrum of L2 is essentially given by the sum of the last two amplitude spectra shown in Fig. 7. As in the case of the unidirectional model, the last intermediate spectrum of the branched model is given by the last amplitude spectrum. However, the scaling is different. Whereas in the sequential model, the amplitude spectrum is multiplied by \( (k_{\text{LL}} + k_{\text{LHR}} - k_{\text{LHR}}')(k_{\text{LL}} + k_{\text{LHR}}) \), using the rate constants of the branched model, in the branched model it is multiplied by \( (k_{\text{LL}} + k_{\text{LHR}} + k_{\text{LHR}}')(k_{\text{LHR}}) \). The former factor amounts to 0.86, explaining the reduced amplitude in Fig. 8, whereas the latter factor has the higher value of 6.1. The large factor is due to the model in which the spectrum is scaled as if all cycling molecules would pass via L2. Therefore, the observed amplitude and intermediate spectra of Fig. 7 and 8 can be well explained by the branched reaction model.
Consequences for the anion transport mechanism

The structure of sHR has clearly demonstrated the chloride binding site in the dark state to be on the extra-cellular side from the Schiff base (Kolbe et al., 2000), and it can be expected that this applies also to pHr, although the binding site appears less restricted. The formation of the L1-intermediate is accompanied by an approach of the anion toward the Schiff base, which had already been concluded from the resonance Raman experiments and is now confirmed by our infrared data. Once this movement has happened, the protein undergoes a conformational change that only involves amide bonds, but not the chromophore, thereby forming the intermediate L2. With respect to time-resolved UV-vis measurements, this transition is spectrally silent and only kinetically apparent as demonstrated for sHR (Váró et al., 1995c) and pHr (Ludmann et al., 2000; Chizhov and Engelhard, submitted).

Because the conformational changes between L1 and L2 are larger than those between L2 and a putative N state, the former transition might reflect the accessibility change of the anion from the extracellular side in L1 to the cytosolic side in L2. A similar interpretation for the two L states has been put forward for sHR (Kolbe et al., 2000), whereas for pHr the change in accessibility has been connected to the L → N transition (Ludmann et al., 2000). However, in view of our different results on the N state, this conclusion may be questionable. The larger amide I changes observed for the O state could reflect the alteration of the binding constant and/or the opening of the channel, enabling the transport of the anion from a site still close to the Schiff base to the cytosolic membrane surface or aqueous phase. In line with this interpretation is the observation that, for pHr, a large electrogenericity is connected with the formation of O (Ludmann et al., 2000). If this view is accepted, the decay of O must involve more molecular events than the mere binding of the anion from the extracellular side. Before this can happen, a switch that restores the original accessibility has to occur. It is possible that the switch has already taken place in O. The observed L2/O equilibrium does not contradict this proposal, because an equilibrium between states of different accessibilities is also observed in bacteriorhodopsin (either L/M2 or M/N). It could, however, also occur in a different O state with altered protein conformation, which, however, is not accumulated due to rapid chloride uptake, but was proposed by Chizhov and Engelhard (submitted). Time-resolved ATR-FTIR investigations may provide further information about such an additional protein state.

We observed an acceleration of the decay of the L/O equilibrium with increasing chloride concentration (5.9 ms at 50 mM, 3.5 ms at 250 mM, and 2.7 ms at 500 mM). A chloride-dependent rate constant from O to HR′ has also been deduced from time-resolved UV-vis measurements (Váró et al., 1995a). One could think that this anion dependence is caused by a rate determined by the recombination with chloride. However, the half-time of 6.2 ms obtained in measurements without chloride is clearly not in agreement with such a fast rate. Here, some other mechanism must restore the initial state.

How can the branching model be incorporated in the anion transport mechanism? As already mentioned, high chloride concentrations (6 M, close to saturation) inhibit the transfer of the anion from the site it occupies in L2 (still close to the Schiff base) to the release site (probably close to the cytosolic surface). The branching will become apparent once the decay of L2 via the L2/O equilibrium is slowed down due to the strongly reduced partitioning of O in the equilibrium. Our own data and recent time-resolved UV-vis measurements (Chizhov and Engelhard, submitted) at varying chloride concentrations demonstrate that in the 0.1- to 2-M concentration range the O amplitude in the equilibrium is strongly dependent on the concentration, and a binding constant of 1 M has been deduced. At saturating NaCl concentration, hardly any O would be observed. It appears that, if the slowing down via the L2/O pathway occurs, the protein can relax via two different pathways, resulting in the branching. The direct relaxation from L2 to HR represents a shortcut in the anion transport mechanism. The anion would be transferred from the L2 site back to the initial binding site. The functional consequence of the other path, leading via L2′ to the dark state, is less clear. The spectra of Fig. 8 demonstrate that the chromophore geometry in both L2 and L2′ are indistinguishable, i.e., a fraction of both contain already an all-trans chromophore. The high anion concentration could cause protonation of the uptake site, although the chromophore is still largely 13-cis. The simultaneous occupation of this site, of the site in L2, and of the release site could cause the large amide changes in L2′. This path would probably lead to netto-anion translocation, because in the transition to the dark state the anion at the site of L2 would finally expel the anion from the release site, due to the fact that the extracellular site with higher affinity in the dark is occupied. The required back-isomerization, either from the L2 or the L2′ states without passing via the O intermediate, could be facilitated by the partial all-trans geometry. We can only speculate about the molecular origin of the altered photoreaction at high NaCl concentration. As suggested by one reviewer, Hofmeister salt effects could play a role. Indeed, as has been shown for bacteriorhodopsin (Der and Ramsden, 1998) and rhodopsin (Vogel et al., 2001), the protein structure also of membrane proteins can be destabilized by high salt concentrations, thereby influencing the photoreaction. Time-resolved infrared experiments on halorhodopsin using strong chaotropic anions, such as thiocyanate, in addition to the transportable anions could test this hypothesis.

It has been shown that high chloride concentration causes an inhibition of chloride transport, which has been ex-
plained by the occupancy of the release site. (Bamberger et al., 1984; Okuno et al., 1999). In the branching model described here, the inhibition of anion transport is caused by the shortcut of the photocycle from the L2 directly to the dark state. If a photocycle that always passes through O is assumed, the high chloride concentration would slow down the photocycle, due to the fact that hardly any O could be formed transiently. The observation of normal millisecond kinetics at saturating chloride concentrations requires a photocycle that does not involve O. Therefore, part of the back-reaction occurs from the L2 state, which results in no netto-transport. The other branch, via L2’, describes a more complicated situation, as it appears possible that ion transport takes place here. It should be emphasized that the spectra of Figs. 7 and 8 are difficult to interpret without branching at L2. However, further experiments using the ATR method (Heberle and Zscherp, 1996), allowing time-resolved measurements under well-defined salt concentrations, are necessary to corroborate these interpretations.

Outlook

It appears important to further investigate the differences between sHR and pHR in relation to their specific amino acid sequences. The correlation of the fine tuning of their properties with the respective structures will lead to a better understanding of these light-driven anion pumps. The observation that the single-atomic anions influence the infrared spectra very little indicates that the anion-protein interaction does not involve the polar residues that have strong infrared intensity. For the binding site in the dark state, this is in agreement with the published structure of sHR. It will be interesting to see whether nitrate also has such a small influence on the spectra. Corresponding investigations are in progress.

The branching model might be important for the transport against a higher membrane potential as it has been studied in the case of bacteriorhodopsin (Nagel et al., 1998). It remains to be seen if the membrane potential affects the futile chloride cycle in which the anion is picked up and released from the same side. Whereas up to now infrared measurements can only be performed under zero voltage conditions, it certainly would be of great interest to understand the mechanism of chloride transport under more physiological conditions.

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