The Chromophore Induces a Correct Folding of the Polypeptide Chain of Bacteriorhodopsin

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ABSTRACT: The p*K* values of the Schiff bases of several bacteriorhodopsin (BR) preparations have been determined by titration. While for the native protein a high p*K* of 13 has been reported [Druckmann et al. (1982) *Biochemistry 21,* 4953], we find that a BR reconstituted from retinal and the apoprotein obtained from the retinal-deficient strain JW5 exhibits a low p*K* value, 8.5. When the retinal chromophore is added to growing JW5 cells leading to in vivo BR formation, this BR shows a high Schiff base p*K*, ≥10.2. A value of 9.3 was determined when BR was reconstituted from retinal and BO, obtained from bleaching BR with hydroxylamine. A low p*K* value of 8.1 was found when 13-trifluoro(CF_3)-retinal was used as chromophore for in vitro reconstitution [Sheves et al. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83,* 3262], which is confirmed in this study. When we add CF_3-retinal to growing JW5 cells, this low p*K* shifts to 9.1. Besides wild-type protein, the apoprotein from the mutant D96N (from the chromophore-deficient strain L-07) was also used for in vitro reconstitution with either chromophore, retinal or CF_3-retinal. Irrespective of the chromophore used, both mutant BRs exhibit low p*K* values of their Schiff bases of 8.1. Flash photolysis with respect to the rise and decay of the M-photocycle intermediate of wild-type and D96N-mutated BR carrying retinal and CF_3-retinal revealed that in both proteins the incorporation of the trifluororetinal leads to a faster rise of the M-intermediate and to a slower decay. Since the apoprotein from the chromophore-deficient JW5 strain of *H. salinarium,* despite its lower boyant density, is arranged into trimers (according to CD measurements), we propose that the high p*K* value of the BR Schiff base is induced by long-distance interactions between BR molecules in the purple membrane patches which control the p*K* of the chromophore.

The membrane protein bacteriorhodopsin (BR)[1] of the archaebacterium *Halobacterium salinarium* functions as a light-driven proton pump and thereby provides an alternative energy source for the bacterium under conditions of low oxygen pressure (*1, 2*). This function of BR is based on its chromophore, all-*trans*-retinal, which is covalently bound to Lys216 of the protein via a protonated Schiff base linkage. Light absorption by the retinal molecule causes an all-*trans* to 13-*cis* double bond photoisomerization within picoseconds (*3*). The energy stored by this process is utilized to drive a series of thermal reactions of chromophore and protein in the micro- and millisecond time range (photocycle of BR), during which a proton is translocated across the cell membrane (*4*).

The positive charge of the Schiff base and interactions of the chromophore with amino acids in its close proximity shift the absorption maximum from 440 nm (λ_{max} of retinal-butylamine PSB in ethanol) into the visible range of the spectrum (λ_{max} of BR: 568 nm). Furthermore, these very specific interactions cause an unexpectedly high value of the p*K* for the Schiff base of 13.0 (*5*). Based on spectroscopic studies of BR mutants, the charged amino acids Asp85 and Asp212 (which are both dissociated in the BR parent state) and Arg82 have been identified as directly interacting with the Schiff base and constituting a complex counterion to the positive charge of the Schiff base (*6*−*8*). It has thus been argued that the precise tuning of the chemical properties of the Schiff base by its protein surrounding is a prerequisite for vectorial proton transport, since it allows variation of the p*K* value by several orders of magnitude when the retinal molecule goes through the photocycle. Such large changes of the p*K* value would make reversible steps (de-/reprotonation) rather unlikely and would thereby—only after conformational changes of the chromophore—allow accessibility of the Schiff base selectively from the inner or the outer part of the proton channel, depending on its high- or low-p*K* state (*9, 10*).

The incorporation of retinal analogues with changed shape or altered electronic properties into the binding site of BR has been used to strengthen or to diminish the influence of the protein surrounding, since altered BR derivatives can be generated, with respect to their absorption maxima and also their proton translocating capability (*11*). Of major impact on the function of BR is the variation of the substituent at position 13 which, e.g., upon changing it into a methoxy group (OCH_3), shifts the absorption to shorter wavelength

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[1] Abbreviations: BR, bacteriorhodopsin; BO, bacterioopsin; CD, circular dichroism; CF_3-BR, BR containing 13-CF_3-retinal as chromophore; SB (PSB), (protonated) Schiff base; WT, wild-type.

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(λ_{max} = 515 nm) and generates a nonfunctional pigment (*12*), whereas the introduction of a trifluoro group at this position (13-CF_3-retinal) generates a strongly red-shifted BR derivative (λ_{max} = 625 nm) which still has proton transporting capability (*13*). In particular, this latter compound has been employed to probe the Schiff base properties, since it was found that—due to the strong electron-withdrawing effect of the CF_3 group—13-CF_3-BR exhibits a remarkably decreased p*K* value of 8.0 (*14*). This result appears quite plausible considering the strong change of the electronic properties in close proximity to the Schiff base nitrogen. In this contribution, we provide evidence that another major factor which regulates the p*K* of the Schiff base is the way the BR sample being investigated is prepared, i.e., whether the chromophore is present during the biosynthesis of the protein, and thus induces a tight folding of the nascent peptide chain around the retinal molecule, or, whether it is added to the already folded apoprotein in which case the packing is much looser.

MATERIALS AND METHODS

Chemical Section. All-*trans*-Retinal was purchased from Aldrich. Retinal solutions for pigment reconstitution were prepared by dissolving small aliquots of the crystalline material in 2-propanol and checking its concentration and purity by UV/VIS spectroscopy and HPLC. HPLC conditions were as previously described (*15*). The synthesis of 13-CF_3-retinal followed the formerly published procedure (*13*) except that ethyl 13-CF_3-retinoate was directly reduced to the aldehyde at −78 °C with DIBAH according to (*16*).

Microbiological and Biochemical Section. H. salinarium strains S9 (BR wild type), JW5 (retinal-deficient mutant), D96N-L33 (D96N mutant), and D96N-L07 (chromophore-deficient D96N mutant) were all provided by D. Oesterhelt and J. Tittor, Max-Planck-Institut für Biochemie, Martinsried, and were grown according to standard protocols (*17, 18*). The same protocols were used for harvesting the cells, cell lysis, and purification of BR or BO, according to the halobacterial strain used, and also for bleaching BR in the presence of hydroxylamine.

Addition of retinal to growing cells was started at the third day after inoculation and was performed every 12 h until harvest after 5 days of growth. Thirty micromoles of retinal was added in every case.

Samples for titration were prepared with 100 mM NaCl and were light-adapted. No significant change of the p*K* value was observed when a sample was kept light-adapted or was treated under dim light conditions. Adjustment of pH and determination of the p*K* value of BRs were performed in 1 × 1 cm cuvettes in volumes of 3 mL. Samples of BR, wild-type or reconstituted, were adjusted to an absorbance of 0.25 at their λ_{max}, and the pH of this sample was determined within the cuvette with a microscale electrode (Mettler-Toledo, Giessen). A UV/VIS spectrum was recorded, and the pH of the solution was altered within the cuvette by the addition of several microliters of 0.1−1 N HCl or NaOH. After allowing for pH stabilization, the new pH value was determined, and an absorption spectrum was recorded. The absorption spectra were corrected for the dilution with acid or base. Titrations were performed with, at minimum, three samples prepared independently. As a further control, the samples were titrated several times to high pH values and back to neutral.

Since acidification of BR causes an increase in scattering due to partial denaturation of the protein, an increase of the base line of the absorption spectra was corrected from the spectrum of a reference (unreconstituted apo-protein). This reference spectrum was adjusted at 400 and 750 nm according to the changes of the spectra of the BR samples upon addition of acid or base. These wavelength positions were chosen since the spectrum of BR has nearly no chromophore absorption at these positions and thus reflects the increase in scattering. The change of the absorption properties as a function of pH was determined from difference spectra, using the absorption spectrum at pH 7 as base line. The change of the absorbance at λ_{max}, determined by this procedure, was plotted against the pH value of the solution. The data points of this plot were fitted by a sigmoidal curve-fitting function which yielded the inflection point of the titration curve.

Sample preparation for CD measurements and detergent treatment of BR for monomerization followed published procedures (*19*). CD spectra were recorded as recently described (*20*).

Physical Section. Samples for flash photolysis were prepared as aqueous solutions and were adjusted to an absorption of 0.2 at their λ_{max}. Measurements were performed at ambient temperature (20 °C) by excitation with a dye-laser system, pumped by a frequency-doubled Nd:YAG laser for CF_3-retinal-containing samples (λ_{ex} = 640 nm), or by the 532 nm light of the frequency-doubled Nd:YAG laser for retinal-containing samples. The laser flash had a duration of ca. 15 ns, and energy of ca. 0.1 mJ per single shot. The chosen energy led to an excitation of between 3 and 8% of the molecules, and so is far from saturation and from generation of secondary photochemistry. A repetition rate of 1 Hz (0.1 Hz for the D96N mutant samples) was chosen, allowing the sample to return to the parent state before the next laser flash. Time-resolved detection of the absorption changes was performed at 420 (retinal-containing preparations) or 430 nm (CF_3-retinal-containing preparations) in the microsecond to second time range. Between 10 and 100 single measurements were averaged and transferred to a VAX-station for further fitting procedures. The absorbance changes were detected with two monitoring light sources: From 100 ns to 20 ms, a pulsed arc lamp was used, whereas for the longer time window up to 10 s, a 100 W dc tungsten halogen lamp was used. For both time windows, the absorbance changes were recorded separately, and combined by computer later. Due to the low light intensity of the pulsed lamp in the short-wavelength range, the microsecond recordings suffer from a low signal-to-noise ratio (see also Figure 4).

RESULTS AND DISCUSSION

(A) pK Values of BR Schiff Bases from Different Preparations. Correlation with Results from the Literature. Titration of BR samples to high pH values led to the formation of an absorption band around 460 nm and an isosbestic point at 510 nm (inset in Figure 1). This absorption is bathochromic compared to the M species of the photocycle (λ_{max} = 410 nm) in which the Schiff base is unprotonated. The 460 nm species has already been reported by Druckmann et al. (1982), who have clearly demonstrated by resonance Raman

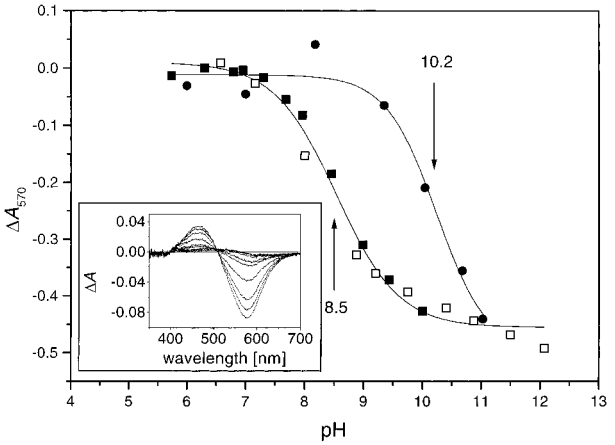

FIGURE 1: Plot of pH-dependent absorption changes of BRs derived from JW5 cells. Curve 1 (inflection point at 8.5), two independently prepared samples (■, □) from the apoprotein from JW5 cells by in vitro reconstitution with retinal (i.e., after isolation from the cells); curve 2 (●, inflection at 10.2), sample prepared by addition of retinal to JW5 cells during growth. Inset: Absorption changes of an in vitro reconstituted sample of JW5 as a function of increasing pH.

spectroscopy that this BR form contains an unprotonated SB. The high p*K* value of 13.3 for native BR reported by Druckmann et al. (*5*) compares well with our experiments. Although we observe increasing denaturation at pH >11, we estimate a lower limit for the p*K* value of ca. 11.5. When CF_3-retinal is used as the chromophore, we determine a pK_a value of 8.0, identically to that described by Sheves et al. (*14*). In our experiments, BO was prepared from white, i.e., retinal-deficient JW5, cells, whereas the sample in the literature (*14*) was made from BO obtained from bleached BR.

(B) pK Values of BRs Using Apoprotein from JW5 Cells. When BO isolated from JW5 cells is reconstituted to BR by the addition of the native retinal chromophore, an unexpectedly low p*K* value of 8.5 is observed (Figure 1). This result is striking, since such a low p*K* has been observed before only when CF_3-retinal was used and was ascribed to the strong electron-withdrawing properties of this chromophore analogue. To further scrutinize this observation that probably the in vitro reconstitution of a retinal-containing BR has a p*K* value different from native BR, we added retinal to JW5 cells during growth; this leads to chromophore incorporation within the growing cell culture. The sample derived from this preparation exhibited lower pH stability than WT BR and showed denaturation at pH >11, making the reading of absorption changes at higher pH values difficult. Accordingly, the data points at higher pH values are not well determined, and the generated fit curve starts to level off around pH 11, yielding an inflection point at pH 10.2 (Figure 1). Due to the experimental problems, this value should be assumed as a lower limit.

The only difference between these two experiments is the addition of the chromophore either during cell growth or after apoprotein preparation. For establishing a Schiff base with a high p*K* value, it is apparently essential that the chromophore is present already during the BR biosynthesis. It can be assumed that the retinal molecule functions as a template for the exact folding of the polypeptide chain which forces the amino acids in its close proximity into a tight

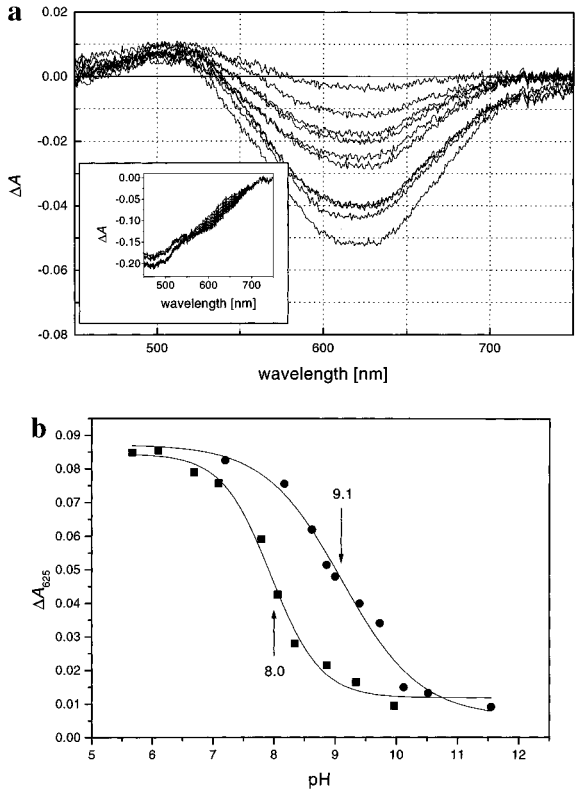

FIGURE 2: (a) pH-dependent absorption changes of in vivo reconstituted CF_3-BR (chromophore added to growing JW5 cells) shown as difference spectra, the absorption spectrum at pH 6.7 serving as reference (base line). The difference spectra shown refer to (from top to bottom) the following pH values: 7.3, 8.2, 8.7, 8.8, 9.0, 9.4, 9.7, 10.1, 10.5, 11.6. Inset: original spectra before base line correction. (b) Determination of p*K* value for CF_3-retinal-containing BRs. (■) Sample prepared in vitro, *i.e.*, by incubation of BO from JW5 cells with the trifluororetinal; (●) sample prepared in vivo by addition of CF_3-retinal during growth of JW5 cells. The inflection point for both experiments indicated by arrows was determined as described in the text.

arrangement whereas the in vitro incorporation of the chromophore leads to a looser packing.

A change of the protein properties upon the addition of the chromophore during growth of the JW5 cells is already evident during the protein preparation. Whereas the apoprotein floats at very low density in the sucrose gradient, the reconstituted-during-growth BR exhibits a much higher density (probably since the presence of the chromophore induces apoprotein biosynthesis) and the protein is found at a similar position in the sucrose gradient as native BR. This observation is well-known (*17, 21*) but has never been investigated in correlation with the Schiff base p*K* value.

When 13-CF_3-retinal is added to growing JW5 cells, the chromophore analogue is incorporated identically as retinal, and the position of the resulting chromoprotein in the sucrose gradient also shifts to a higher density. Although the BR analogue is formed in much lower yield due to the low chemical stability of the CF_3-retinal in aqueous solution, the red-shifted absorption with respect to native BR is readily seen (λ_{max} = 625 nm, Figure 2a). When this chromoprotein is titrated, the p*K* value of the Schiff base is higher by more than 1 unit (p*K* of 9.1) than for a sample prepared in vitro (Figure 2b).

(C) Control Experiments. The finding that the Schiff base p*K* depends on the origin of the BR and the sample

Table 1: Absorption Maxima and p*K* Values of the Schiff Bases of BR Preparations Described in the Text

sample	absorption maximum (nm)	Schiff base p*K* value
wild-type BR	568	13.3[a], >11.5[b,c]
wild-type BR + 2-propanol (20 μL, control experiment)	568	>13.0
in vivo incorporation of retinal into JW5 cells	565	>10.2[c]
in vitro incorporation of retinal into BO from JW5 cells	565	8.5
incorporation of retinal into BO from hydroxylamine-bleached BR	565	9.3
in vivo incorporation of CF_3-retinal into JW5 cells	625	9.1
in vitro incorporation of CF_3-retinal into BO from JW5 cells	625	8.0[a], 8.0[b]
in vitro incorporation of retinal into BO from D96N L07 cells	565	8.1
in vitro incorporation of CF_3-retinal into BO from D96N L07 cells	625	8.1

[a] Literature values. [b] This work. [c] Reading of absorbance changes was impeded by increased scattering due to partial denaturation, see text.

preparation is quite unexpected and required a number of control experiments in order to elucidate the possible influence of experimental parameters. Repeated titrations with the same sample and also experiments with separately prepared samples led to deviations by less than 0.2 pH unit. The good correlation between two independently performed experiments is demonstrated in Figure 1 (open and filled symbols) for the data from in vitro-reconstituted JW5 samples.

Since the spectral properties of BR can be strongly influenced by added salt, all samples were prepared with 100 mM NaCl, and the effect of the salt which is generated by the addition of acid or base to change the pH was taken into account. The amounts of acid and base applied during titration could at most double the initial salt concentration. A control experiment starting with a 200 mM salt concentration showed no change of the absorption properties or the p*K* of the Schiff base. Even when the same sample was titrated several times to high pH and back to neutral, no significant change of the resulting p*K* value was observed.

It should furthermore be pointed out that the variation of the p*K* of reconstituted BRs is not due to the addition of small aliquots of 2-propanol as the solvent for retinal. A sample of native BR when supplemented with 20 μL of 2-propanol still exhibits a high p*K* value of >13.0 (extrapolated, see Table 1).

(D) CD Spectroscopy of BR Derived from JW5 Cells. The well-known low density of BO originating from JW5 cells has been related to the loss of crystallinity which is characteristic for native BR embedded in purple membrane patches, since the apoprotein derived from JW5 cells, and also the reconstituted protein, does not diffract in the X-ray beam (*21*). Thus, this protein has often been described as a kind-of monomerized BR for which—possibly due to the missing constraints of the neighboring protein molecules—a different arrangement of the helical segments might result. It was of interest for the studies of the binding site properties presented here to know whether the structure of JW5-derived BR is monomeric or whether it still shows quasi-crystalline properties despite the loss of X-ray diffraction. A method well-suited for this kind of investigation is circular dichroism (CD) spectroscopy. The CD spectrum of native BR in purple membrane in the region of the absorption band consists of two bands of opposite signs and different amplitudes, viz., a positive band at 535 nm and a much weaker negative band at 600 nm. This biphasic spectrum is observed only for the intact purple membrane; when the membrane is solubilized, e.g., by treatment with detergents, the CD spectrum becomes monophasic positive and matches the UV/vis spectrum (*22*, *23*). According to one line of reasoning, electronic interaction of the retinal chromophores in the ordered purple membrane gives rise to delocalized exciton states and the typical split CD spectrum. Upon solubilization of the membrane, the ordered structure is lost, and exciton interaction is no longer possible. The observed, altered CD reflects the inherent and/or environment-induced chirality of the monomeric chromophores.

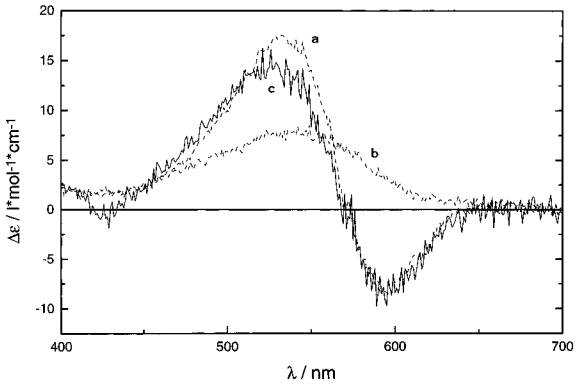

FIGURE 3: CD Spectra of various BR forms. (a) Reconstituted BR, derived from incubation of JW5-derived BO with retinal; (b) as (a), but after addition of detergent (7 mM Triton X-100) which leads to a loss of membrane structure; (c) CD spectrum of native BR.

The CD spectrum of a JW5-derived BR which was produced by in vitro reconstitution, i.e., addition of retinal after apoprotein preparation, is biphasic and nonconservative and is practically identical to the CD spectrum of native BR (Figure 3). Like BR in purple membrane, this CD spectrum changes into that of monomeric BR upon detergent treatment.

(E) BR Formation with BO from Hydroxylamine-Bleached BR. Besides from JW5 cells, BO can be prepared by bleaching BR in the presence of hydroxylamine. In this procedure, the chromophore is trapped in the form of its stable oxime derivative, and the binding site of the apoprotein remains intact and the pigment can be reconstituted with retinal to form functional BR. Using a BO sample from such a preparation for the reconstitution with retinal leads to BR with a p*K* of the Schiff base of 9.3, which again deviates markedly from that of native BR (titrations not shown). We conclude that bleaching alters the protein structure with respect to the arrangement of the amino acids in the chromophore binding site as well.

(F) pK Values of D96N Mutants. Aspartate-85 and aspartate-96 have been identified as residues being essential for the proton translocation of BR during the de- and reprotonation of the Schiff base in the L to M and the M to

N transitions of the BR photocycle (*24*, *25*). Since mutations of these residues drastically change the photocycle kinetics, it may be assumed that the p*K* values of the Schiff bases of these mutants also vary from that of the wild-type protein. Unfortunately, the titration of D85T to high pH values turned out to be impossible due to the instability of this mutant, which led to denaturation.

D96N apoprotein can be obtained like the WT protein from a retinal-deficient *H. salinarium* strain (L-07) which has similar properties as JW5-derived BO. In vitro reconstitution of this apoprotein with either retinal or CF_3-retinal yields chromoproteins with absorption maxima identical to the corresponding wild-type proteins ($\lambda_{max} = 570$ nm for retinal and 625 nm for CF_3-retinal). Again as observed for the JW5-derived wild-type BO, these D96N mutants exhibit p*K* values of 8.1 for both chromophores (Table 1).

(G) Flash Photolysis of Retinal and CF_3-Retinal-Containing BRs. Since the JW5-derived pigments exhibit differences in their Schiff base acidity, it was of interest to determine whether this variation might also give rise to altered photocycle kinetics. The reconstituted BRs (obtained from incubation of the apoproteins, either derived from JW5 or, in the case of D96N mutants, derived from L07 cells, with retinal or CF_3-retinal) were subjected to flash photolysis. In the context of this work, we concentrate only on the detection of the rise and the decay of the M intermediates of the reconstituted BRs which is directly related to the de- and reprotonation of the Schiff base.

For native BR, which was used as a reference, the rise of the M form can be fitted with two exponentials of ca. 65 and 155 μs. In addition, a very short-lived component of ca. 1 μs (5% of the total amplitude) is observed. For the decay of the M intermediate, two lifetimes of 7.2 and 15.4 ms are required with relative contributions of ca. 60 and 40% to the total signal amplitude. Comparable data were determined for reconstituted BR with JW5-derived apoprotein. Here, rise kinetics of 50 and 122 μs and decay components of 5.9 and 17.9 ms were determined from the fit (for the relative amplitudes, see Table 2). The data for CF_3-BR vary from those of the retinal-containing samples: The M intermediate, which exhibits a red-shifted absorption maximum (430 nm), is formed faster (4 and 30 μs) and persists for longer times (1.8 and 32 ms) than the M intermediate of native BR. These changes in the kinetics concur well with the proposal that the increased electronegativity of the 13-CF_3 group facilitates the deprotonation and hinders the reprotonation. The observation that the reconstituted and native BR samples with different p*K* values both exhibit similar M kinetics eludes an explanation. The origin of the difference may be that the titration of the Schiff base is performed in a static regime whereas the situation after flash excitation of the BR sample encompasses the dynamics of the photocycle reactions. It may thus be speculated that the photoisomerization of the chromophore generates a similar arrangement of chromophore and protein for both samples (i.e., the native and the in vitro reconstituted BR) irrespective of their origin.

Flash photolysis of the D96N mutants, containing either retinal or CF_3-retinal, yields for both chromophores the extended lifetime of the M form formerly described for retinal which is ascribed to the mutation: The retinal-containing sample is generated with two kinetics of 18 and 163 μs, and decays with the described retarded kinetics of several hundred milliseconds. For a sufficient fit, three exponentials with $\tau = 3.1$, 56, and 392 ms are required. Similar to the wild-type protein, the CF_3-D96N BR shows a faster formation of the M intermediate (4 and 80 μs), and

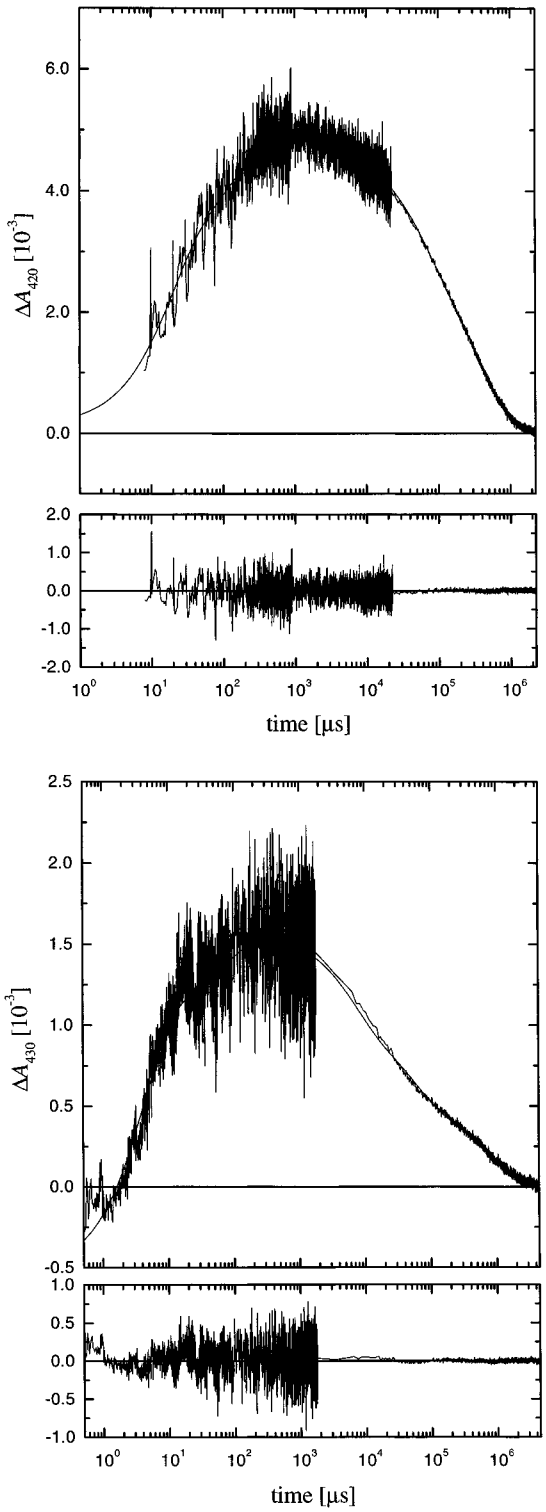

FIGURE 4: Rise and decay kinetics of (top) retinal-D96N BR and (bottom) CF_3-D96N BR. Absorption changes at 420 nm (retinal-containing sample) and at 430 nm (CF_3-retinal-containing sample) are shown in a semilogarithmic plot. Due to different detection-light intensities (see Materials and Methods), the scatter in the short time range is relatively large. The absorption changes are well fitted by two rise and three decay components (residues between flash photolysis signals and fit are shown at the bottom of the figure).

Table 2: Rise and Decay Kinetics of the M Intermediates of BR Preparations Described in the Text

sample	formation of M intermediate (μs)	relative amplitudes (%)	decay of M intermediate (ms)	relative amplitudes (%)
wild-type BR	$\tau_1 = 1$, $\tau_2 = 65$, $\tau_3 = 155$	5, 55, 40	$\tau_1 = 7.2$, $\tau_2 = 15.4$	63, 37
BO from JW5 cells + retinal	$\tau_1 = 50$, $\tau_2 = 122$	45, 55	$\tau_1 = 5.9$, $\tau_2 = 17.9$	73, 27
BO from JW5 cells + CF_3-retinal	$\tau_1 = 4$, $\tau_2 = 30$	67, 33	$\tau_1 = 1.8$, $\tau_2 = 32$	45, 55
BO from D96N L07 cells + retinal	$\tau_1 = 18$, $\tau_2 = 163$	60, 40	$\tau_1 = 3.1$, $\tau_2 = 56$, $\tau_3 = 392$	4, 34, 62
BO from D96N L07 cells + CF_3-retinal	$\tau_1 = 4$, $\tau_2 = 80$	76, 24	$\tau_1 = 6.4$, $\tau_2 = 55$, $\tau_3 = 870$	31, 38, 31

an even slower decay than the retinal-containing sample (6.4, 55, and ca. 870 ms, Figure 4).

CONCLUSION

In conclusion, we find that a remarkable change of the Schiff base p*K* of BR can be effected by different modes of preparation, depending on whether the retinal chromophore is present during cell growth (in vivo reconstitution) or is added only after the apoprotein preparation (in vitro reconstitution). When present during the biosynthesis of the protein, the retinal chromophore of BR appears to induce a very specific packing of the protein which is manifest in the high p*K* of its Schiff base. The unexpected low p*K* of the JW5-derived protein may be related to its loss of crystallinity. However, it still exhibits the excitonic CD spectrum (Figure 3), which is comparable to that of native BR and demonstrates that this mutant still forms an ordered structure in the membrane. If the trimeric structure of native BR is also the smallest unit in the JW5-derived protein, the packing of these trimers over wide distances, as encountered in the purple membrane, could force the BR molecules into a specific conformation which shifts the p*K* to the observed high value.

Flash photolysis of the wild-type and D96N mutants of BR revealed that in both proteins the electron-withdrawing property of CF_3-retinal promotes the deprotonation of the Schiff base (M formation) and delays its reprotonation (M decay).

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