Conversion of Bacteriorhodopsin into a Chloride Ion Pump

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In the light-driven proton pump bacteriorhodopsin, proton transfer from the retinal Schiff base to aspartate-85 is the crucial reaction of the transport cycle. In halorhodopsin, a light-driven chloride ion pump, the equivalent of residue 85 is threonine. When aspartate-85 was replaced with threonine, the mutated bacteriorhodopsin became a chloride ion pump when expressed in *Halobacterium salinarium* and, like halorhodopsin, actively transported chloride ions in the direction opposite from the proton pump. Chloride was bound to it, as revealed by large shifts of the absorption maximum of the chromophore, and its photointermediates included a red-shifted state in the millisecond time domain, with its amplitude and decay rate dependent on chloride concentration. Bacteriorhodopsin and halorhodopsin thus share a common transport mechanism, and the interaction of residue 85 with the retinal Schiff base determines the ionic specificity.

Bacteriorhodopsin (*1*) and halorhodopsin (*2*) are active, light-driven electrogenic pumps for protons and chloride ions, respectively, in the cell membranes of halobacteria. Both contain seven transmembrane helical segments, and their chromophores consist of retinal linked to a lysine residue by means of a protonated Schiff base near the middle of helix G. The sequence similarity of the two proteins has raised expectations (*3*) that the mechanism of ion transport might be similar in the two systems, even though one translocates a cation and the other an anion in opposite directions.

The active site of bacteriorhodopsin is an ion pair formed by the protonated Schiff base and the anionic Asp^{85} residue (*4*). Transfer of a proton from the Schiff base to Asp^{85} initiates not only proton release to the extracellular side and several other proton transfers that follow, but also a protein conformational shift (*5*) that is associated with a change in the access of the Schiff base (*6*) from the extracellular toward the cytoplasmic side and the proton donor Asp^{96}. In halorhodopsin, the residue that corresponds to Asp^{85} is threonine, which is conserved in various species (*7*). Lacking a proton acceptor, the Schiff base does not become deprotonated in halorhodopsin. As expected, replacement of Asp^{85} with Asn abolished Schiff base deprotonation in bacteriorhodopsin also, as well as the light-driven proton transport (*6*, *8*); however, in the D85N and D85T mutants (*9*) currents associated with two-photon reactions in films attached to planar lipid bilayers suggested the possibility of more complex modes of proton translocation (*10*). Likewise, the related retinal protein, sensory rhodopsin I of halobacteria, becomes a proton pump in the absence of its transducing protein, but not when the Asp residue that corresponds to residue 85 is replaced with Asn (*11*).

The difference of the ion specificities of bacteriorhodopsin and halorhodopsin is intriguing. The chloride ion binds near the Schiff base of halorhodopsin (*12*). It is remarkable, therefore, that at pH below 2, where Asp^{85} is permanently protonated, chloride causes a blue-shift of the maximum peak of bacteriorhodopsin. Chloride affected the photocycle differently from bromide, whereas the effects of H_2O and D_2O were equivalent, and in oriented membranes a net photocurrent was observed. It was proposed (*13*) that when Asp^{85} is protonated bacteriorhodopsin might bind and transport chloride like halorhodopsin, but there was no conclusive evidence that chloride, rather than proton, was transported. Mutants of bacteriorhodopsin with decreased negative charge in the extracellular region also exhibited halide-dependent absorption shifts, which suggests that the anions bind to the positively charged Schiff base (*14*). In the D212N mutant, the normal photocycle was observed only in the presence of chloride, apparently because the anion contributes to the Schiff base counterion (*15*). However, this protein still transports protons rather than chloride. For this reason, we felt that the difference between the two pumps is not only a coulombic effect from the lesser negative charge near the Schiff base of halorhodopsin, but also the result of differing hydrogen bonding geometries of Asp and Thr at position 85 that may be of importance for chloride binding at this location and its transport.

Chloride transport by halorhodopsin is usually assayed by examination of passive proton uptake in the presence of chloride and an uncoupler (*16*). Light indeed caused a net inward transport of protons in D85T bacteriorhodopsin–containing membrane vesicles (*17*) that was facilitated by uncoupler and abolished by TPP^+ (tetraphenyl phosphonium bromide), a membrane-permeant cation (Fig. 1A). Halorhodopsin also exhibits such transport, but the D85T gene was expressed in *H. salinarium* Pho81 cells that lack all bacterial opsins (*18*). The observed chloride affinity, in the molar range (Fig. 2A), also confirms the absence of chloride transport by halorhodopsin in these assays, because this would have reached a maximal rate near a concentration of chloride of 150 mM (*19*), at which almost no transport was seen. The absence of halorhodopsin was further confirmed by the observation (*15*) that the pH changes were unaffected by heating at 55°C, a treatment that inactivates halorhodopsin but not bacteriorhodopsin (*20*). The simple relation of the initial rate versus the light intensity (Fig. 1B) demonstrates that the transport of chloride is driven by a single-photon reaction.

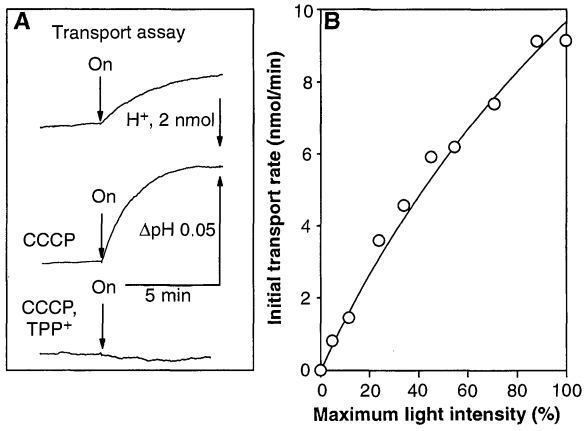

Fig. 1. Light-driven transport of chloride ions by D85T bacteriorhodopsin in cell envelope vesicles in 4 M NaCl. The onset of illumination (with yellow light, >530 nm) is indicated with arrows labeled "On." The reversals of the pH changes after the light was turned off are not shown. The pH was 6.9, and the vesicles in the assay (*25*) contained 0.2 nmol of D85T bacteriorhodopsin. (**A**) Demonstration of passive proton uptake. The pH rise is more rapid in the presence of 5 μM CCCP (carbonyl cyanide *m*-chlorophenyl hydrazone), and 1 mM TPP^+ abolishes the pH rise. (**B**) Dependence of the initial rate of transport on light intensity.

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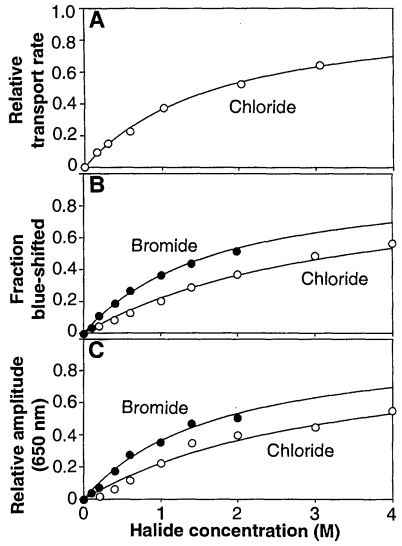

Fig. 2. Dependency on halide of various measured properties of D85T bacteriorhodopsin. (**A**) Initial rate of passive proton uptake into D85T bacteriorhodopsin–containing cell envelope vesicles in the presence of the uncoupler CCCP. This is as shown in Fig. 1A, except that 1.5 M Na_2SO_4 and 3 M NaCl were mixed for the titration. (**B**) Binding of halide, as reflected in the spectral shifts in Fig. 3A, but at pH 6. (**C**) Amplitude of maximal absorption change at 650 nm at about 1.5 ms after photoexcitation, as shown in Fig. 4. All amplitudes are relative to the calculated maximal values at infinite halide concentration.

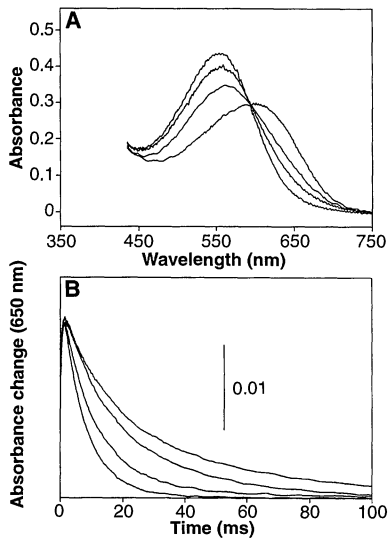

Fig. 3. (**A**) Spectral shift of D85T bacteriorhodopsin in the presence of chloride. Buffer, 50 mM phosphate, pH 6.5. NaCl concentrations: 0 (1 M Na_2SO_4), 1.4 (plus 0.3 M Na_2SO_4), 3.0, and 4.0 M, in the direction of increasing absorbance near 560 nm. (**B**) Decay of the red-shifted photointermediate formed after 1.5 ms, as shown in Fig. 4, at different chloride concentrations. Conditions were as in (A) but at pH 6. The amplitudes are scaled to the highest peak for comparison. A relative size bar is shown. Chloride concentrations were 1.4, 2.0, 3.0, and 4.0 M, in the direction of increasing decay rates.

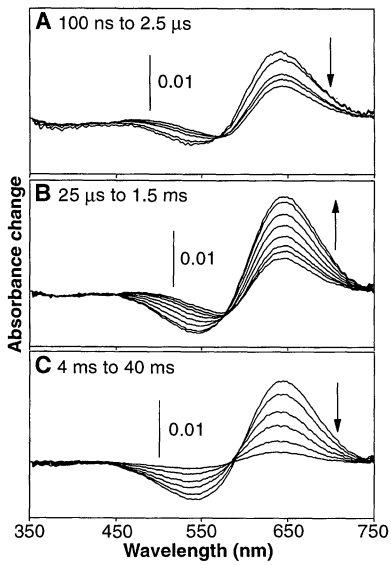

Fig. 4. Spectral changes after the photoexcitation of D85T bacteriorhodopsin in 2 M NaBr and 50 mM phosphate (pH 6). The photochemical reaction was initiated with a 7-ns Nd-YAG (yttrium-aluminum-garnet) laser flash, and the resulting difference spectra were measured with an optical multichannel analyzer as described (*28*). The delay times between the laser pulse and the measurements are 100 ns, 400 ns, 1 μs, 1.5 μs, and 2.5 μs in (**A**), 25 μs, 100 μs, 150 μs, 250 μs, 400 μs, 600 μs, 1 ms, and 1.5 ms in (**B**), and 4 ms, 6, ms, 10 ms, 15 ms, 25 ms, and 40 ms in (**C**). Relative size bars for absorbance are shown in each panel.

The existence of chloride transport implies that chloride will bind to D85T bacteriorhodopsin. Near neutral pH, chloride and bromide caused a large shift in the spectrum toward shorter wavelengths, from 600 to 556 and 566 nm, respectively (Fig. 3A), which is similar to the effect in halorhodopsin from *Natronobacterium pharaonis* (*21*), although dissimilar to that in *H. salinarium*, where the shift is toward longer wavelengths (*22*). The apparent binding constant is in the molar range (Fig. 2B), whereas the dissociation constant (K_d) for chloride binding in *N. pharaonis* halorhodopsin is 1 mM (*21*, *23*). The D85N mutant exhibits no halide-dependent spectral shifts under these conditions (*24*). Halides affect also the photochemical cycle of D85T. The first photointermediate of the blue form of D85T (in 1 M Na_2SO_4) is a red-shifted state (identified as K in the wild-type photocycle), which decays within a few microseconds (*24*). Thereafter, blue-shifted intermediates appear that have lower extinctions than that of the unphotolyzed state, like the L and N states in the photocycles of wild-type "acid blue" bacteriorhodopsin at pH 2 or D85N (*6*). In 2 M NaBr (or NaCl), these blue-shifted intermediates are followed by a red-shifted state that appears with a 0.5-ms rise time (Fig. 4). Its decay repopulates the initial state.

This effect is very similar to what has been reported for halorhodopsin (*21*, *25*). Although we recently attributed the late red-shifted photoproduct of halorhodopsin from *H. salinarium* to an artifact that arises from the chromophore not being 100% trans (*26*), such a red-shifted intermediate does arise at the end of the photocycle of all-trans halorhodopsin from *N. pharaonis* (*21*, *23*). As in this halorhodopsin, the chloride (and bromide) dependence on the amplitude of absorption change, which is a result of the red-shifted intermediate shown in Fig. 2C, is consistent with the halide binding equilibria (Fig. 2B). The photointermediate thus appears to arise from the halide-binding forms. The absence of a change in absorption near 400 nm (Fig. 4) indicates that the Schiff base does not deprotonate in this photocycle.

The decay rate of the red-shifted state is linearly dependent on the concentration of chloride (Fig. 3B). This is also true for *N. pharaonis* halorhodopsin (*23*) and suggested that this rate is limited by the uptake of chloride in the same way as the recovery of the initial wild-type bacteriorhodopsin state depends on proton uptake (*27*). However, in bacteriorhodopsin the proton uptake is on the cytoplasmic surface, whereas in halorhodopsin the equivalent chloride uptake would have to be on the extracellular surface. The binding of the chloride near the Schiff base in this reaction, as required by such a mechanism, is consistent with the blue-shift of the maximum as the initial state recovered. Thus, in the way the pH-dependent properties of wild-type bacteriorhodopsin had provided clues to the proton transport, these chloride-dependent properties common to halorhodopsin and D85T bacteriorhodopsin begin to suggest a chloride transport mechanism.

Our results show that upon replacement of Asp^{85} with Thr (but not Asn), bacteriorhodopsin is converted into a chloride transport system and shares all properties relevant for chloride transport with halorhodopsin from *N. pharaonis* but with a high affinity for binding the chloride. This finding implies that except for the ion specificity conferred by residue 85, the mechanisms of proton and chloride transport must be the same. It is difficult to visualize this unless the interaction of the Schiff base and residue 85, and its changes upon photoisomerization of the retinal, are the essential elements of the transport (*6*).

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The TBP-TFIIA Interaction in the Response to Acidic Activators in Vivo

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A yeast TBP mutant (N2-1) is described here that is defective specifically in responding to acidic activators in vivo. N2-1 does not support activation by Gal4, Ace1, and Gcn4, but appears unaffected for constitutive transcription, repression by the Cyc8-Tup1 and Not complexes, and transcription by polymerase I (Pol) and Pol III. In vitro, N2-1 fails to interact with TFIIA, but it associates normally with a TATA element, an acidic activation domain, and TFIIB. Fusion of the small subunit of TFIIA to N2-1 restores activation function in vivo. Thus, an efficient interaction between TBP and TFIIA is required for transcriptional activation in vivo.

RNA Pol II transcription is regulated by gene-specific activator proteins that interact with enhancer elements located distal to the site of transcription initiation. Activators generally contain a DNA-binding domain and a separable activation domain (or domains) that stimulate the transcription machinery. Many activation domains are characterized by a large proportion of acidic amino acids. Such acidic activation domains function in a wide variety of eukaryotic species, which is indicative of a highly conserved mechanism of transcriptional activation.

Most models for activation invoke protein-protein interactions between activators bound at enhancer elements and the general transcription factors assembled at the TATA and initiation elements (*1*). These general factors include the TATA-box binding protein (TBP), TBP-associated factors (TAFs) that are components of the Pol II–specific TFIID complex, as well as TFIIA, -B, -E, -F, -H, -J, and Pol II (*2*). In vitro, activation domains can directly interact with TBP (*3*), TAF110 and TAF40 (*4*), TFIIA (*5*), TFIIB (*6*), and TFIIH (*7*). Biochemical studies suggest that some of these interactions are important for transcriptional activation in vitro (*4*, *8–10*). In addition, several TBP mutants that support basal but not activated transcription in vitro have been described; one of these is specifically defective for interaction with TFIIB, whereas the others show combinations of defects in interactions with an acidic activation domain (VP16), TFIIA, TFIIB, or DNA (*11*). The significance of these interactions to the mechanism of activation in vivo is unknown.

Recruitment of TBP to the promoter in vivo can be a rate-limiting step for transcription that is enhanced by acidic activator proteins (*12*). Transient transfection experiments in mammalian cells indicate that TBP plays a role in transcriptional activation (*13*, *14*). Thus, interactions that facilitate the association of TBP with the TATA element are potential targets for acidic activators. However, little is known about which interactions are critical for the response to acidic activators in vivo. Here, we report the isolation and characterization of a TBP mutant protein (N2-1) whose properties indicate that the TBP-TFIIA interaction is essential for transcriptional activation by acidic activator proteins.

TBP is highly conserved throughout eukaryotic evolution (*15*) and is essential for transcription by all three nuclear RNA polymerases in vivo (*16*). The N2-1 derivative was isolated in a genetic screen for TBP derivatives that are competent for Pol III transcription but have another functional defect. Specifically, a set of complex and compact libraries of yeast TBP mutant proteins (*17*) was screened for derivatives that complemented the temperature-sensitive phenotype of TBP-F155S (where Phe^{155} was changed to Ser), a Pol III–specific TBP mutant (*17*), and also conferred ts growth when present as the sole source of TBP (Fig. 1A). In addition, the strain with N2-1 as the only TBP grew slowly even at 30°C (Fig. 1B), and the cells exhibited a large and elongated morphology. This phenotype was not the result of reduced expression or instability of the mutant TBP, because immunoblot analysis of the mutant strain indicated amounts of TBP equal to those found in the wild type (Fig. 1C). Moreover, the structure of the N2-1 protein was not substantially compromised, because N2-1 was normal for Pol III transcription at the restrictive temperature (Fig. 1D).

To characterize the transcriptional properties of the N2-1 protein, we determined RNA levels for various genes in a strain with this derivative as the only TBP. Wild-type levels of Pol II transcription from the *DED1*, *HIS3*, *RPS4*, *TBP*, and *TRP3* genes occurred in cells grown at the permissive or restrictive temperature (Fig. 2, A and B). In

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