The Residues Leu 93 and Asp 96 Act Independently in the Bacteriorhodopsin Photocycle: Studies with the Leu 93→Ala, Asp 96→Asn Double Mutant

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ABSTRACT Previous mutagenesis studies with bacteriorhodopsin have shown that reprotonation of the Schiff's base is the rate-limiting step in the photocycle of the D96N mutant, whereas retinal re-isomerization and return of the protein to the initial state constitute the rate-limiting events in the photocycle of the L93A mutant. Thus, in the D96N mutant, decay of the M intermediate is slowed down by more than 100-fold at pH 7. In the L93A mutant, decay of the O intermediate is slowed down by ~250-fold. We report here that in the L93A, D96N double mutant, decay of the M intermediate, as well as the formation and decay of the O intermediate, are slowed down dramatically. The photocycle is completed by the decay of a long-lived O intermediate, as in the L93A mutant. The decay of the M and O intermediates in the double mutant parallels the behavior seen in the single mutants over a wide temperature and pH range, arguing that the observed independence is an intrinsic property of the mutant. The slow decay of the M and O intermediates can be selectively and independently reversed under conditions identical to those used for the corresponding intermediates in the D96N and L93A single mutants. Because the effects of the two individual mutations are preserved in the double mutant and can be independently reversed, we conclude that residues Asp 96 and Leu 93 act independently and at different stages of the bacteriorhodopsin photocycle. These results also show that formation of the O intermediate only requires protonation of the Schiff's base and is independent of the protonation of Asp 96 from the aqueous medium.

INTRODUCTION

Bacteriorhodopsin is a membrane protein that functions as a light-driven proton pump. Upon light absorption, retinal, which is covalently linked to Lys 216, is isomerized from the all-*trans* to the 13-*cis* configuration, and the resulting protein conformational changes trigger the release of a proton from the Schiff's base into the extracellular medium. At physiological pH, the release of a proton into the extracellular medium coincides with deprotonation of the Schiff's base (Grzesiek and Dencher, 1986). At least three distinct stages are involved in the regeneration of the initial bacteriorhodopsin state at or near neutral pH: i) reprotonation of the Schiff's base by Asp 96, ii) proton uptake from the cytoplasmic medium leading to the reprotonation of Asp 96, and iii) protein-mediated, thermal re-isomerization of retinal from the 13-*cis* to the all-*trans* configuration, together with return of both retinal and the protein to the starting bacteriorhodopsin state. The coupling between retinal re-isomerization and proton uptake is thought to be central to the successful completion of the photocycle (Fodor et al., 1988).

Spectroscopic measurements have shown that formation and decay of a series of optical intermediates (J, K, L, M, N, and O) provide convenient markers for the protein and retinal structural changes that occur during the photocycle. In particular, spectroscopic studies of site-specific mutants in which the kinetics of proton uptake, or retinal re-isomerization, are strongly affected have provided useful insights into the molecular mechanisms underlying the late stages of the photocycle (Lanyi, 1993; Ebrey, 1993; Krebs and Khorana, 1993; Subramaniam et al., 1991; Brown et al., 1994). When Asp 96 is replaced by Asn (D96N mutant), the photocycle is slowed down by ~100 to ~500-fold at pH 7 compared to wild-type bacteriorhodopsin, because of accumulation of the M intermediate (Otto et al., 1989; Butt et al., 1989; Miller and Oesterhelt, 1990). The decay of this M intermediate kinetically corresponds both to the rate of proton uptake and to the rate of reprotonation of the Schiff's base. The kinetic defect in the D96N mutant can be overcome either by lowering the pH, which promotes direct reprotonation of the Schiff's base from the aqueous phase, or by the addition of azide, which appears to act as a catalyst for the reprotonation of the Schiff's base (Tittor et al., 1989; Otto et al., 1990; le Coutre et al., 1995).

A similar profound effect is observed upon replacement of Leu 93 by Ala (L93A mutant), in which completion of the photocycle is slowed down by ~250-fold compared to wild-type bacteriorhodopsin because of the accumulation of a long-lived, red-shifted intermediate (Subramaniam et al., 1991). We have assigned this intermediate to be the O intermediate based on three features that it shares with the O intermediate in the wild-type photocycle: the red shift in λ_{max} relative to the initial bacteriorhodopsin state, the observation that its formation occurs on the time scale of decay of the M intermediate, and the observation that it is in equilibrium with an N-like intermediate. Spectroscopic studies show that the kinetic defect in the photocycle of the

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L93A mutant occurs at a stage after the completion of proton transport and can be overcome in the presence of strong background illumination, thus by-passing the slow thermal decay of the O intermediate (Delaney et al., 1995). Time-resolved retinal extraction experiments demonstrate the continued presence of a 13-*cis* intermediate in the photocycle of the L93A mutant during the lifetime of the long-lived O intermediate. Together, these observations lead to the conclusion that an interaction between Leu 93 and the C-13 methyl group of retinal is important for completion of retinal re-isomerization and resetting of the protein to its initial state (Fig. 1).

These observations, as well as previous studies with wild-type bacteriorhodopsin (bR), can be understood in terms of the following model of the late stages of the photocycle, which incorporates all intermediates in models previously proposed by Lozier et al. (1975), Lanyi (1993), and Ebrey (1993), as well as a 13-cis O intermediate first proposed by Milder (1991) on theoretical grounds.

$$\underset{\text{13-}cis}{M} \leftrightarrows \underset{\text{13-}cis}{N^{-1}} \leftrightarrows \underset{\text{13-}cis}{N^{0}} \leftrightarrows \underset{\text{13-}cis}{O} \leftrightarrows \underset{\text{twisted all-}trans}{O} \rightarrow \underset{\text{all-}trans}{bR.}$$

In the model for wild-type bacteriorhodopsin, M decay occurs with the donation of a proton from Asp 96 to the Schiff's base. Proton uptake from the cyctoplasm occurs during the N^{-1} to N^0 transition to reprotonate Asp 96. Resonance Raman studies have found retinal configuration in the N intermediate to be 13-*cis* (Fodor et al., 1988), whereas retinal configuration in the O intermediate was found to be a conformationally distorted all-*trans* retinal form (Smith et al., 1983). Thus isomerization has been proposed to occur during the N^0 to O transition.

The studies of the L93A mutant lead to two possible models for its photocycle. In one scenario, retinal re-isomerization is the rate-limiting step of the photocycle leading to the accumulation of 13-*cis* N and 13-*cis* O intermediates, which are in equilibrium. Alternatively, the rate-limiting step could involve the completion of retinal re-isomerization and the resetting of the protein, which would lead to the accumulation of 13-*cis* N and conformationally distorted all-*trans* O intermediates. Independently of which O intermediate accumulates, the optically detected transient at $\lambda > 600$ nm provides a convenient marker for steps that occur subsequent to proton uptake.

To further investigate the roles of Asp 96 and Leu 93 in the late stages of the bacteriorhodopsin photocycle, we have measured kinetic and thermodynamic parameters of the photocycle in the L93A, D96N double mutant and compared them with measurements for the single mutants. Some aspects of the photocycle of the L93A, D96N double mutant under strong background illumination, and in the presence of azide were previously discussed in the report by Tittor et al. (1994). Here, we demonstrate that the two mutations act independently in the double mutant and can be independently "rescued" under the same conditions used for the corresponding single mutants. Furthermore, they also suggest that retinal re-isomerization as well as any subsequent protein conformational changes that occur during the decay of the O intermediate must be coupled to (i.e., require) protonation of the Schiff base and are independent of the protonation of Asp 96 from the aqueous medium.

MATERIALS AND METHODS

Construction and purification of mutants

Purple membranes were isolated using standard procedures (Oesterhelt and Stoeckenius, 1974) from halobacterial strains expressing the L93A mutant (Delaney et al., 1995), the D96N mutant (Krebs et al., 1993; kindly provided by Drs. M.P. Krebs and H.G. Khorana), and the L93A, D96N double mutant (Tittor et al., 1994; kindly provided by Drs. J. Tittor, U. Schweiger, and D. Oesterhelt). Purified membranes were suspended in 0.15 M KCl, 10 mM phosphate buffer at pH 7 and an optical density (O.D.) of ~0.5 at 538 nm.

Transient absorption spectroscopy

Transient absorbance measurements were carried out as previously described (Delaney et al., 1995). A 10-μs flash at 550 nm (~0.1 mJ/cm^2 per flash) from a xenon lamp was used to initiate the photocycle, and a weak probe beam was used to follow the subsequent absorbance changes. In a typical experiment, 16 transient absorption traces were averaged from a sample volume of 1 ml. The decay of the M intermediate was followed at 405 nm. The formation and decay of the O intermediate were followed at 600 or 625 nm for the L93A, D96N double mutant and at 605 nm for the L93A single mutant. Absorbance changes at 483 nm were used to follow changes in concentration of the initial bacteriorhodopsin state as well as the N intermediate. Because the decay of the N and O intermediates corresponds kinetically to the formation of the initial bacteriorhodopsin state (Cao et al., 1993; Ames and Mathies, 1990; Delaney et al., 1995), the slow component at 483 nm represents the sum of absorbance changes due to decay of the N and O intermediates (decrease in absorbance with time) and recovery of the initial bacteriorhodopsin state (increase in absorbance with time). A net increase in absorbance is observed at this wavelength because the contribution from the O intermediate to the absorbance at 483 nm is negligible, and because the extinction coefficient of bacteriorhodopsin is higher in comparison to the N intermediate (Lozier et al., 1975).

The source of the continuous background illumination was a 150-W slide projector whose light was filtered with a red ($\lambda > 620$ nm) cut-on filter. This wavelength range allows preferential illumination of the O intermediate while minimizing excitation of the M intermediate, which can also be photoconverted to bacteriorhodopsin (Ohno et al., 1983). The intensities used were well within the linear range previously determined for acceleration of photocycling time in the L93A mutant (Delaney et al., 1995). Under these conditions, the acceleration in time required to complete the photocycle can be modeled as simply being due to light absorption by the long-lived N and O intermediates.

Proton kinetics measurements

Kinetics of proton release and uptake were measured in 0.15 M KCl as described (Grzesiek and Dencher, 1986) by taking the difference between absorbance transients at 460 nm recorded on samples with and without the pH indicator dye pyranine (8-hydroxyl-1,3,6-pyrene trisulfonate; Sigma), present at a concentration of ~35 μM.

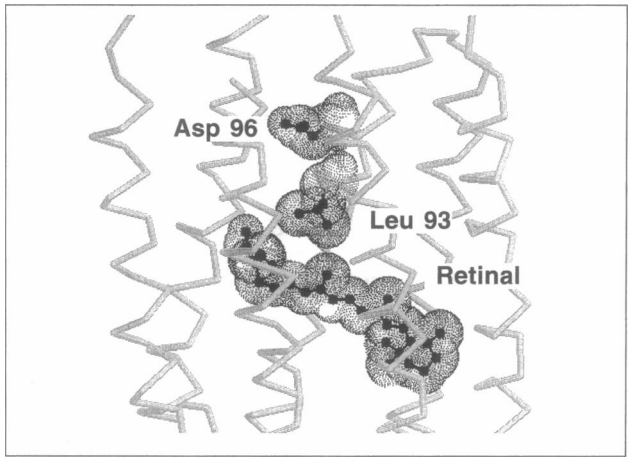

FIGURE 1 Location of Leu 93 and Asp 96 in bacteriorhodopsin, based on the atomic structural model determined by Henderson et al. (1990). In the late stages of the photocycle, the Schiff's base is reprotonated by proton transfer from Asp 96, which, in turn, is reprotonated by proton transfer from the cytoplasm. The interaction of retinal with Leu 93, located one turn of an α-helix below Asp 96, is important in the thermal re-isomerization of retinal to the all-*trans* state and the resetting of the protein.

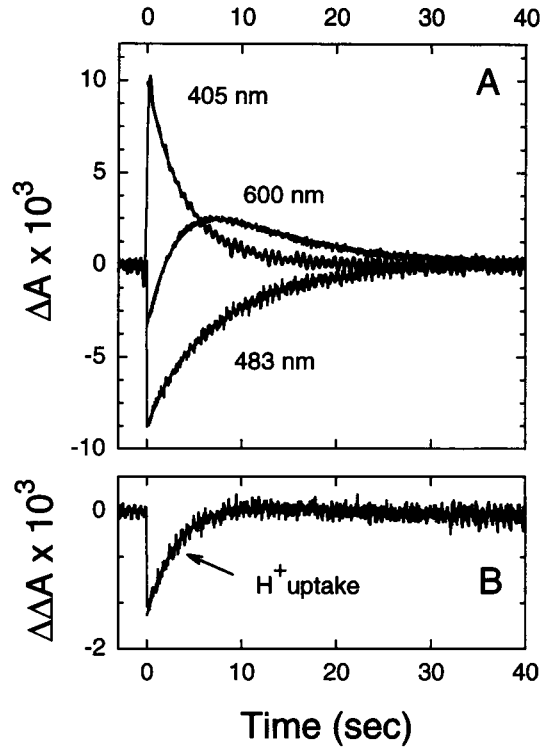

FIGURE 2 Transient absorption changes after flash excitation of membrane fragments of the L93A, D96N double mutant in 0.15 M KCl with 10 mM phosphate buffer at pH 7. (*A*) Absorbance changes at 405 nm and 600 nm show changes in concentration of the M and O intermediates, respectively. The biphasic changes at 483 nm primarily reflect the sum of absorbance changes resulting from formation and decay of the N intermediate (on the same time scale as formation and decay of the O intermediate), and from repopulation of the initial bacteriorhodopsin state (on the same time scale as the decay of the N and O intermediates). (*B*) Kinetics of light-induced proton uptake from the aqueous phase (0.15 M KCl) as determined by the absorption changes of the pH indicator dye pyranine. The uptake kinetics are similar to the time scales observed in *A*, both for decay of the M intermediate and for formation of the O intermediate.

RESULTS

Photocycle kinetics in the L93A, D96N double mutant

Transient absorbance measurements show that in the double mutant, decay rates of the M and O intermediates are ~700-fold and ~1500-fold slower, respectively, than the corresponding rates observed for wild-type bacteriorhodopsin at pH 7. As previously observed in the case of the D96N single mutant (Butt et al., 1989), the formation of the M intermediate was slightly slower (time constant of ~0.24 ms) than that observed in wild-type bacteriorhodopsin. The decay of the M intermediate was biphasic (Fig. 2 *A*). The major component (~85% of the total amplitude) decayed with a time constant of 3.5 s at pH 7, and the minor component decayed with a time constant of ~10 s. Absorbance measurements at 605 nm under the same conditions showed that the rates of formation and decay of the O intermediate respectively matched the time constants observed for the fast and slow rates of decay of the M intermediate. The time constant for decay of the O intermediate is ~6-fold longer than that observed in the L93A mutant. Transient absorbance measurements at 483 nm to monitor recovery of the initial bacteriorhodopsin state also showed a biphasic recovery. The time constant for the faster component of the recovery at 483 nm was the same as that observed for decay of the fast component of the M intermediate. Similarly, the time constant for the slower component of the recovery at 483 nm was the same as that observed for decay of the O intermediate. These data establish that the photocycle of the double mutant can be adequately described by a simple model in which the O intermediate exists in equilibrium with the M intermediate and is formed by decay of the M intermediate.

To determine the relationship between the photointermediate kinetics and proton uptake in the double mutant, the kinetics of pH changes in the aqueous phase were measured using the pH indicator dye pyranine. Previous studies have shown that in the D96N mutant, proton uptake occurs with the decay of the M intermediate (Marinetti et al., 1989; Otto et al., 1989), and that in the L93A mutant, proton uptake occurs with formation of the O intermediate (Delaney et al., 1995). Fig. 2 *B* shows that in the L93A, D96N double mutant, the kinetics of proton uptake are similar to those for the decay of the M intermediate and for the rise of the O intermediate. (The low resolution of the pH trace only allows detection of the main component of proton uptake, which we associate with the decay of the fast component of the M intermediate.) The absorbance measurements and the proton kinetics strongly suggest that in the double mutant, the formation of the long-lived O intermediate is kinetically coupled to proton uptake from the aqueous medium. To-

gether, these observations demonstrate that the effects of the individual mutations, i.e., slow decay of the M intermediate due to the D96N mutation, and slow decay of the O intermediate due to the L93A mutation, are preserved in the double mutant.

pH dependence of the photocycle of the L93A, D96N double mutant

To further test whether the two mutations in the double mutant act independently, we took advantage of the finding that the decay of the O intermediate is pH independent in the L93A mutant (Fig. 3 *A*), whereas the decay of the M intermediate is accelerated with decreasing pH in the D96N mutant (Fig. 3 *A* and Tittor et al., 1989). As shown in Fig. 3 *B*, the rate of formation of the O intermediate in the double mutant was decreased by >30-fold upon raising the pH from 5.5 to 8. An identical kinetic effect (Fig. 3 *B*) was observed for the rate of decay of the fast component of the M intermediate. Over the same pH range, the rate of decay of the O intermediate changed by <1.5 fold. The corresponding rates in the single mutants show that the observed pH dependence of decay of the M intermediate in the double mutant is similar to that seen for the D96N single mutant, and that the pH dependence of decay of the O intermediate in the double mutant is similar to that observed for the L93A single mutant (Fig. 3, *A* and *B*). The pH dependence in the range (pH 5.5–pH 7.5) of decay of the M and O intermediates in the double mutant can therefore be explained in terms of a superposition of effects observed in the photocycles of the D96N and L93A mutants. Above pH 7, the contribution from the slow component of decay of the M intermediate increases sharply, and the decay of the absorbance at 405 nm due to the M intermediate begins to approach the time required for completion of the photocycle, thus making an accurate determination of the time constants of decay of the fast and slow components difficult. This is attributed to the increased accumulation of the slow component of decay of the M intermediate, and to the approach of the time constant for decay of the fast component to that of the slow component.

Effects of background illumination and azide on photocycling time in the L93A, D96N double mutant

Next we determined whether the kinetic defects in decay of the M and O intermediates in the double mutant could be independently reversed, as in the case of the L93A and D96N single mutants. Light-induced transient absorbance changes were determined in the absence and presence of red background illumination, which accelerates the photocycle of the L93A mutant (Fig. 4, *A–C*). Measurements of the rate of ground state re-population show that in the presence of red background illumination, the photocycle of the double mutant was accelerated by ~5-fold (compare Fig. 4, *A* and *B*), and the time required for completion of the photocycle was close to that observed for the rate of decay of the M intermediate (compare Fig. 4, *B* and *C*). Under these conditions, the photocycle of the double mutant approached that of the photocycle of the D96N mutant; i.e., M decay was the rate-limiting step of the photocycle. We conclude that in the presence of red background illumination, the slow thermal decay of the O intermediate is overcome by light-driven conversion of the N and/or O intermediates to the initial bacteriorhodopsin state. As previously observed in the case of the L93A mutant (Delaney et al., 1995), retinal extraction experiments demonstrated the continued

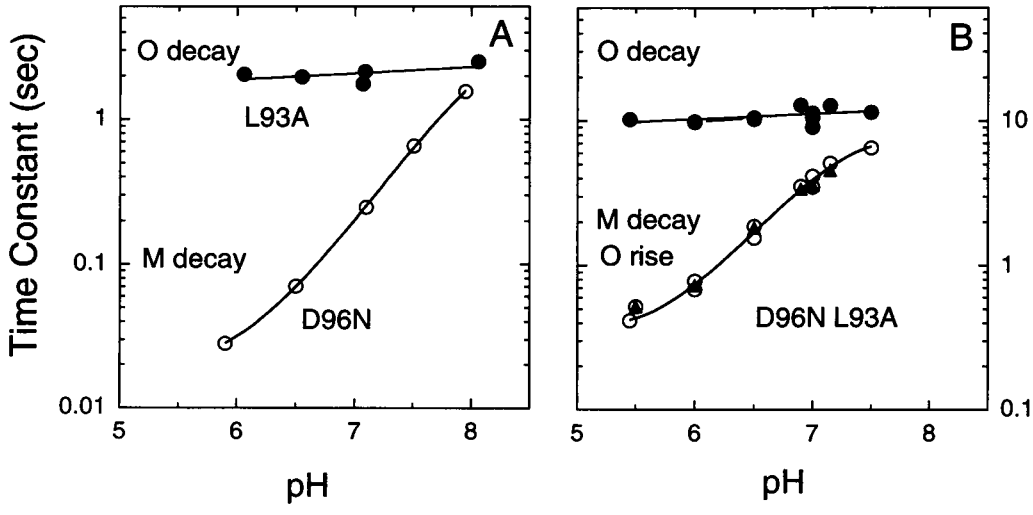

FIGURE 3 pH dependence of the rate of decay of M and O intermediates in the L93A mutant, the D96N mutant, and the L93A, D96N double mutant. (*A*) Time constants for decay of the M intermediate (○) in the D96N mutant in the presence of trace amounts of azide (~0.3 mM) and decay of the O intermediate (●) in the L93A mutant. (*B*) Time constants for formation (○) and decay (●) of the O intermediate in the L93A, D96N double mutant. The time constants for the fast-decaying component of the M intermediate (▲) match those for the formation of the O intermediate (○). At pH > 7.5, no accumulation of the O intermediate was observed, consistent with the decay of the M intermediate being the rate-limiting step.

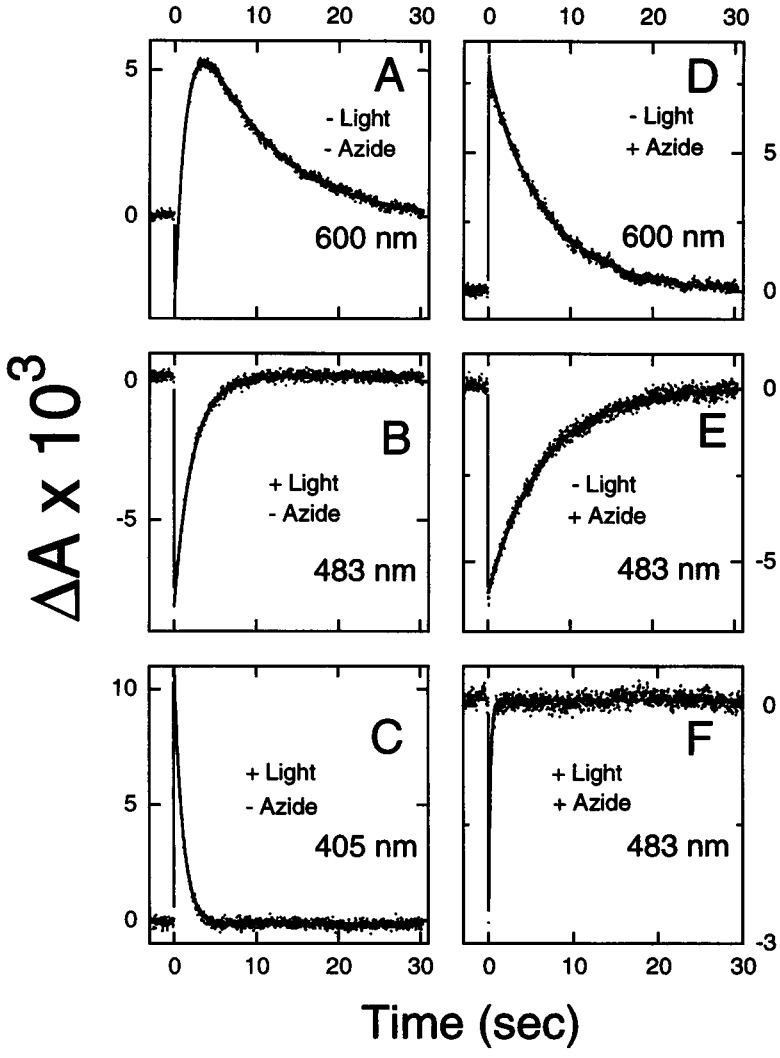

FIGURE 4 Effects of red background illumination and sodium azide on the photocycle of the L93A, D96N double mutant at pH 6.5. (*A*) Absorbance changes at 600 nm, showing formation and decay of the O intermediate in the absence of both background illumination and azide. (*B* and *C*) Absorbance changes in the presence of red background light ($\lambda > 620$ nm) measured at 483 nm (*B*), showing recovery of the initial bacteriorhodopsin state, and at 405 nm (*C*), showing decay of the M intermediate. The photocycling rate is accelerated by fivefold under these conditions. (*D* and *E*) Absorbance changes in the presence of 4 mM azide measured at 600 nm (*D*), showing decay of the O intermediate, and at 483 nm (*E*), showing recovery of the initial bacteriorhodopsin state. The addition of azide accelerates formation of the O intermediate but does not affect the decay of the O intermediate or the time required for completion of the photocycle. (*F*) Absorbance changes in the presence of red background light and 4 mM azide measured at 483 nm, showing recovery of the initial bacteriorhodopsin state in ~0.24 s. In the presence of both orange and red background illumination ($\lambda > 560$ nm), a photocycling time of ~100 ms was observed, close to the value reported for the L93A mutant under similar conditions (Delaney et al., 1995).

presence of a 13-*cis* intermediate in the photocycle at a time when most of the M intermediate has decayed (data not shown).

To test whether the decay of the M intermediate in the double mutant could be selectively accelerated as in the D96N mutant, we determined the effect of azide on the photocycle. At pH 6.5, the addition of 4 mM azide accelerated formation of the O intermediate by ~300-fold, without affecting the rate of decay of the O intermediate (Fig. 4, *A* and *D*). As previously reported by Tittor et al. (1994) and confirmed here, the presence of azide also accelerated the decay of the M intermediate by the same extent (data not shown). Measurements of rate of ground state re-population showed that the kinetics of recovery of the initial bacteriorhodopsin state matched the kinetics of decay of the O intermediate (compare Fig. 4, *D* and *E*). Under these conditions, the photocycle of the double mutant resembled that of the L93A mutant with respect to the fast rise and slow decay of the O intermediate; in addition, the slow decay of the M intermediate could be overcome by the addition of azide.

To test whether the slow decay of the M and O intermediates could be simultaneously "rescued," photocycle kinetics were measured in the presence of 4 mM azide and red background illumination (Fig. 4 *F*). Under these conditions, decay of the M intermediate as well as the rate of ground state re-population (by photoconversion of the N/O intermediates) were accelerated. These results demonstrate that the L93A and D96N mutations not only exert their respective effects on the kinetics of decay of the M and O intermediates independently, but that the resulting kinetic defects can also be independently reversed.

Temperature dependence of the photocycle of the L93A, D96N double mutant

To demonstrate that the observed kinetic independence of decay of the M and O intermediates was not merely a consequence of the conditions chosen for the measurements, the temperature dependence of the decay of the M intermediate and of the formation and decay of the O

intermediate was determined (Fig. 5). At all temperatures, the rate observed for the fast component of decay of the M intermediate matched the rate of formation of the O intermediate. From these measurements, an activation energy of ~35 kJ/mol for decay of the M intermediate was determined (at pH 7), which is close to the value of 32 kJ/mol reported for the decay of the M intermediate in the D96N single mutant (at pH 7; Miller and Oesterhelt, 1990). The activation energy observed for the rate of decay of the O intermediate in the double mutant was determined to be ~80 kJ/mol (Fig. 5). This value is close to the ~77 kJ/mol observed in the case of the L93A single mutant (Delaney et al., 1995). The addition of azide did not alter the activation energy for decay of the O intermediate in the double mutant. These observations demonstrate that the addition of the L93A mutation does not significantly alter the activation barrier for decay of the M intermediate when only the D96N mutation is present, and that addition of the D96N mutation does not significantly alter the activation barrier observed for decay of the O intermediate when only the L93A mutation is present.

DISCUSSION

The experiments described here show that long-lived M and O intermediates are observed in the photocycle of the double mutant. All of the experimental observations can be adequately explained by the photocycle scheme previously mentioned for the late stages of the photocycle of wild-type bacteriorhodopsin and the L93A mutant. The decay of the M intermediate in the double mutant parallels the behavior seen in the D96N mutant up to pH ~7. Under these conditions, the decay of the M intermediate is dominated by the fast component. Over this pH range, decay of the O intermediate in the double mutant is pH independent, as in the L93A mutant. At high pH values (e.g., at pH 8), decay of the M intermediate becomes the rate-limiting step, and as a result, the O intermediate is not populated. Moreover, because the decay of both M and O intermediates parallels that of the single mutants over a wide temperature range (at pH 7), it follows that the observed independence is an intrinsic property of the double mutant. Consistent with this independence, the slow decay of the M and O intermediates could be selectively and independently reversed under conditions identical to those used for the corresponding intermediates in the D96N and L93A single mutants. Because the effects of the two individual mutations are preserved in the double mutant, and can be independently reversed, we conclude that the residues Asp 96 and Leu 93 independently affect the decay of the M and O intermediates and thus act at different stages of the bacteriorhodopsin photocycle.

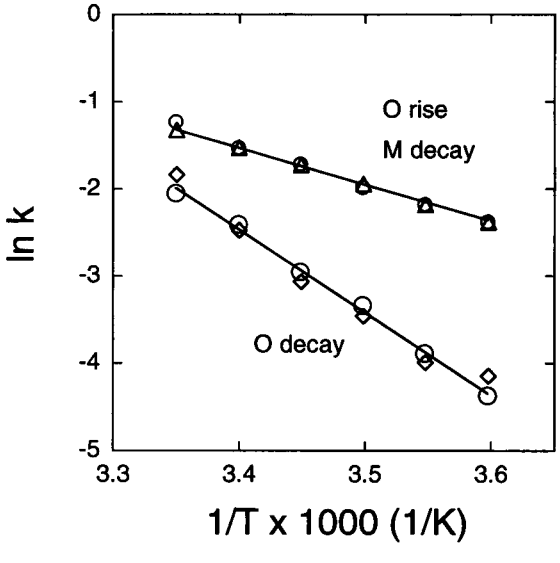

FIGURE 5 Arrhenius plots of the temperature dependence (25°C to 5°C) of the kinetics of decay of the M intermediate and of the rise and decay of the O intermediates in the L93A, D96N double mutant at pH 7. *Upper trace:* △, decay of the M intermediate; ○, rise of the O intermediate. *Lower trace:* ○, decay of the O intermediate; ◇, decay of the O intermediate in the presence of 4 mM azide. The plot shows that between 25°C and 5°C, the rates of decay of the M intermediate are identical to those observed for rise of the O intermediate, and the rate of decay of the O intermediate is unaffected by the addition of azide, which accelerates the rate of formation of the O intermediate.

Analysis of the photocycle of the double mutant also highlights the connections between protonation of Asp 96, protonation of the Schiff's base, and the formation of the N and O intermediates. First, because a long-lived O intermediate is observed, it follows that protonation changes at Asp 96 are not required for either the formation or the decay of the O intermediate. Second, because the decay of the O intermediate is not affected by changes in the rate of protonation of the Schiff's base (e.g., in the absence and presence of azide, or at different pH values), the molecular events associated with decay of the O intermediate must be separate from those involved in protonation of the Schiff's base. Finally, as previously pointed out by Otto et al. (1990), the observation of long-lived intermediates subsequent to the M intermediate establishes that there is an absence of N and O intermediates in the photocycle of the D96N mutant only because decay of the M intermediate is the rate-limiting step, and not because of an intrinsic difference in the sequence of events in the photocycle introduced by the D96N mutation.

The independence of the D96N and L93A mutations is consistent with the different mechanisms that have been proposed for the roles of Asp 96 (as an internal proton donor) and Leu 93 (as a key van der Waals contact residue with retinal) in the photocycle (Tittor et al., 1989; Otto et al., 1989; Delaney et al., 1995). However, because Asp 96 and Leu 93 are located within one turn of a helix from each other, it is conceivable that the presence of the D96N mutation may have an indirect effect on van der Waals interactions of Leu 93 with retinal and other residues in its vicinity. An interesting feature of the double mutant is that the time constant for decay of the O intermediate is about 6 times higher than that observed in the L93A single mutant over a wide temperature range. One explanation for the rate

effect could be that local perturbations such as hydrogen bonding and/or changes in the arrangements of nearby structural water molecules induced by the D96N mutation may alter the local structural rearrangement associated with conversion of the O intermediate to the initial bacteriorhodopsin state.

Electron and x-ray diffraction analysis of intermediates in the photocycle of wild-type bacteriorhodopsin and in the D96G mutant have shown that there are significant structural changes in the latter stages of the photocycle (Koch et al., 1991; Subramaniam et al., 1993). Structural changes at the stage of the photocycle in which the Schiff's base and Asp 96 become protonated appear to be predominantly localized to the cytoplasmic domain (Subramaniam et al., 1993). In wild-type bacteriorhodopsin, because retinal reisomerization as well as proton uptake from the medium are believed to occur with the formation of the O intermediate, it is not possible to clearly distinguish between structural changes that are involved in proton uptake versus those that are involved in subsequent steps involving retinal reisomerization and return of the protein to its initial conformation. However, in the L93A and L93A, D96N mutants, the time scales for protonation of Asp 96 and the Schiff's base are well separated kinetically from later events in the photocycle, for which the decay of the O intermediate provides a convenient marker. Our experiments suggest that distinct protein conformational changes must occur in the photocycle subsequent to protonation of the Schiff's base, a hypothesis that can be tested by high-resolution structural studies of intermediates in the photocycle of the L93A and L93A, D96N mutants.

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