Effects of individual genetic substitutions of arginine residues on the deprotonation and reprotonation kinetics of the Schiff base during the bacteriorhodopsin photocycle

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ABSTRACT The rates are determined for the deprotonation and reprotonation of the protonated Schiff base (PSB) as well as of formation and decay of the UV transient in the photocycle of seven bacteriorhodopsin (bR) mutants in which Arg-7, 82, 164, 175, 225, or 227 are replaced by glutamine and Arg-134 by cysteine. The results show that all these mutations increase the rate of deprotonation of the PSB compared to ebR, (wild-type bacteriorhodopsin expressed in *Escherichia coli*) greatly increase the rate of the reprotonation of the SB (Schiff base) in the case of the Arg-164 and Arg-175 mutations and dramatically decrease this rate in the case of the Arg-227 mutation. Temperature studies on the latter mutant suggest that the observed change in its rate of reprontonation is due to large decrease in the energy and entropy of activation, similar to those observed for Asp-96 mutations (Miller, A. and D. Orsterhelt. 1990. *Biochim. Biophys. Acta.* 1020:57–64). These results suggest that the reprotonation process is changed to a proton diffusion-controlled mechanism in the Arg-227 mutant due to a change in the structure of the proton channel. The absorption intensity ratio (A_{UV}/A_Mslow) of each arginine mutant relative to that of ebR is found to be similar to that for native purple membrane (PM) except for the Arg-227 mutant where it is greatly reduced, and for the Arg-82 mutant where it is not observed, suggesting that both Arg-227 and Arg-82 residues somehow play roles in inducing the UV transient absorption. All the above results are discussed in terms of the model for the structure of bR proposed by Henderson, R., J. M. Baldwin, T. A. Ceska, F. Zemlin, E. Beckmann, and K. H. Downing. (1990. *J. Mol. Biol.* 213:899–929).

INTRODUCTION

Bacteriorhodopsin (bR) in the purple membrane of *Halobacterium halobium* is a retinal-based, light-transducing pigment (1). It contains an all *trans* retinal bound to Lys-216 and light absorption through a protonated Schiff base (PSB) takes place which initiates a photocycle of a number of intermediates (2–4):

$$bR_{568} \xrightarrow[400fs]{h\nu} J_{625} \xrightarrow[5ps]{} K_{610} \xrightarrow[2\mu s]{} L_{550} \xrightarrow[60\mu s]{+H^+} M_{412} \xrightarrow[ms]{-H^+} N_{520} \rightarrow O_{640} \rightarrow bR_{568}$$

This results in the deprotonation of the PSB during the L → M step and reprotonation of the SB in the M → N step. As a result, protons are pumped from the inside to the outside of the *H. halobium* cell, creating an electrochemical proton gradient used by the cell for metabolic processes under anaerobic conditions. Though the function of bR is clear, the detailed molecular mechanism of proton pumping still needs much more elucidation.

The primary sequence of bR has been established (5, 6) and the technique of recombinant DNA allows specific amino acid substitutions in the bR molecule (7–10), creating mutants. By studying these mutants, it is possible to explore the role of specific amino acid residues in proton translocation (11–13).

One type of mutants we have studied is the arginine mutants. There are a total of seven arginine residues in the bR molecule. By mutagenesis, each of the arginine residue has been individually replaced by the neutral glutamine (Gln) or cysteine (Cys) (14, 15). Previous studies on these seven mutants indicate that no individual arginine residue serves to define the lengths of the membrane-embedded helices and that no single electrostatic interaction involving an arginine residue represents the main stabilizing forces that retain the folded structure of the protein (14). Meanwhile, the Arg-175 mutant showed a marked decrease in chromophore regeneration rate, suggesting a possible involvement of this residue in the folding process (14). The Arg-227 mutant exhibited both reduced proton-pumping activity and dramatically decreased rate of M decay, indicating that this residue probably facilitates a conformational change during the proton-pumping cycle, which brings a donor residue close to the SB (14, 16, 17). The Arg-82 mutant showed alterations in both chromophore regeneration and proton-pumping activity which are highly pH dependent, with pK_a near neutral. It was suggested that this residue serves to stabilize the ionized form of another residue, possibly Asp-85 which itself protonates

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0006-3495/91/07/172/07 $2.00

at pH 3.5, causing transition to the blue membrane (14, 18).

In our present work, in addition to monitoring the rise and decay of the M intermediate, we also measured the corresponding kinetics of the UV transient. Whereas it is quite a well established fact that the M intermediate is associated with deprotonation and reprotonation of the PSB, the identity of the UV transient has been quite controversial. Earlier researchers had assigned it to tyrosinate formation (19–21). Lately, this assignment has been questioned. Raman (22, 23) and NMR (24) results do not suggest tyrosinate formation during the bR photocycle. A possible alternative assignment is a perturbed tryptophan absorption (22). If so, it should be due to the formation of a charge which is very likely a weak acid with a pK_a near 9 as demonstrated by FTIR results (25), which suggest that some aspartic acids in bR could have pK_a values around 9.

By monitoring the rise and decay of the M intermediate at 405 nm and the UV transient absorption at 296 nm, we show that whereas the decay rate of the M intermediate is greatly enhanced for the Arg-164 and Arg-175 mutants, it is dramatically decreased in the case of the Arg-227 mutant. It is interesting that the Arg-164 and Arg-175 mutants have such an increased rate for reprotonation of the SB even though neither Arg-164 nor Arg-175 is located in the proton channel according to the model of Henderson et al. (26). The observation that the Arg-227 mutant shows a decreased rate for reprontonation of the SB is in agreement with the previous finding (14). Except for the Arg-82 and Arg-175 mutants, the decay of the UV transient also exhibited a similar trend, although not as pronounced. Furthermore, the absorption of the UV transient formed relative to that of the slow rising M intermediate for the arginine mutants is found to be similar to that of ebR and native PM, except for the Arg-227 and Arg-82 mutants where it is reduced to ~30% and zero, respectively. Temperature dependence study of the slow-decaying component of the M intermediate shows that whereas there is no significant difference between native PM and wild-type ebR in terms of activation energy and entropy, the corresponding activation energy and especially the entropy are drastically decreased in the case of the Arg-227 mutant. This observed effect is consistent with the latest model by Henderson et al. (26) in which Arg-227 is located on the inner surface of bR and is very likely to interact with Asp-96 which has been identified as the internal proton donor in the reprotonation of the SB during the bR photocycle (17, 27, 28). The absence of such an interaction could lead to a change in the mechanism of the reprotonation process of the SB upon changing the charged Arg-227 to the neutral Gln in the Arg-227 → Gln mutation.

MATERIALS AND METHODS

Preparation of wild-type bR (ebR) and its arginine mutants have been described previously (14,15). The proteins were reconstituted in vesicles with polar lipids from *H. halobium*, buffered at pH 6 (except the Arg-82 mutant for which the pH was adjusted to 8.0 with Tris buffer for kinetic measurements). Master slants of ET 1001 strain of *H. halobium* were generously supplied to us by R. Bogomolni (University of California, Santa Cruz) and W. Stoeckenius (University of California, San Francisco). Native PM was purified from *H. halobium* by a combination of the methods described by Oesterhelt and Stoechkenius (29) and Becher and Cassim (30).

The experimental set up for obtaining the transient absorption at 405 and 296 ± 5 nm is basically the same as described previously (31, 32), except that the data acquisition and analysis were done by a personal computer. The extraction of the rate constant for both the rise and the decay of the M intermediate and the UV transient absorption were done by using a nonlinear least-square fitting computer program assuming biexponential rise or decay, with variable preexponential factors as shown below. For rise kinetics,

$$A^{\text{rise}}(t) = 1 - C^{\text{fast}}e^{-k^{\text{fast}}t} - (1 - C^{\text{fast}})e^{-k^{\text{slow}}t},$$

where $A^{\text{rise}}(t)$ is the absorbance of the rising transient at time t, C^{fast} and $(1 - C^{\text{fast}}) = C^{\text{slow}}$ are fractions of the total absorbance at $t = \infty$ having rate constant k^{fast} and k^{slow} respectively. For decay kinetics,

$$A_{\text{decay}}(t) = C_{\text{fast}}e^{-k_{\text{fast}}t} + (1 - C_{\text{fast}})e^{-k_{\text{slow}}t},$$

where $A_{\text{decay}}(t)$ is the absorbance of the decaying transient at time t, C_{fast} and $(1 - C_{\text{fast}}) = C_{\text{slow}}$ are fractions of the total absorbance at $t = 0$ having rate constant k_{fast} and k_{slow}, respectively.

The signals were averaged for 1,000 shots. Each independent acquisition of transient absorption profile was repeated at least twice. The photolyzing laser was pulsed at a repetition rate ranging from 1 to 0.1 Hz depending on the recovery rate of the transient species being measured.

RESULTS AND DISCUSSION

By visual inspection, the Arg-7, 134, 164, 225, and 227 mutants all show the characteristic purplelike color of the ebR and native PM chromophore at pH 6. However, at the same pH, the Arg-175 mutant is more pinkish than purple and the Arg-82 mutant is blue. The former observation is very likely a reflection of the Arg-175 residue being important in the proper folding pathway of the bR molecule (14), whereas the latter observation is, as pointed out previously (14), due to the presence of a red-shifted inactive species (the blue form) which can be converted back to the active form (the purple form) by increasing pH.

Table 1 shows the kinetic parameters of the rise of the M intermediate and the UV transient in the photocycle of native PM, ebR, and the arginine mutants. The slow component of the UV transient absorption corresponds to protein changes whereas the fast component is due to absorption changes resulting from its retinal (32, 33), and hence only the slow component is shown. As

TABLE 1 The rate constants*‡ for the formation of the M intermediate and the UV transient at room temperature (~22°C)

Protein	k ($10^4 s^{-1}$)		
	M^{fast}	M^{slow}	UV transient
Native bR	8 (0.14)	1.4 (0.86)	1.3 (0.45)
ebR	10 (0.10)	1.2 (0.90)	1.0 (0.51)
Arg-7 → Gln	31 (0.34)	7.8 (0.66)	7.1 (0.33)
Arg-82 → Gln (pH 8)	32 (0.76)	6.1 (0.24)	Not observed
Arg-134 → Cys	33 (0.38)	8.2 (0.62)	6.3 (0.25)
Arg-164 → Gln	38 (0.22)	5.4 (0.78)	5.1 (0.20)
Arg-175 → Gln	34 (0.29)	6.3 (0.71)	0.5 (0.40)
Arg-225 → Gln	24 (0.31)	5.1 (0.69)	4.4 (0.23)
Arg-227 → Gln	29 (0.27)	6.2 (0.73)	4.0 (0.20)

*Each value shown is an average of at least two independent measurements. The relative difference between the independent measurements is < 10%.

‡In parentheses are the relative amplitudes for the two M components and the slow fraction of the total absorbance change of the UV transient.

TABLE 2 The rate constants*‡ for the decay of the M intermediate and the UV transient at room temperature (~22°C)

Protein	k ($10^2 s^{-1}$)		
	M_{fast}	M_{slow}	UV transient
Native bR	9.5 (0.40)	1.6 (0.60)	1.3 (0.81)
ebR	5.3 (0.31)	1.5 (0.69)	1.7 (0.80)
Arg-7 → Gln	19. (0.18)	1.7 (0.82)	1.2 (0.84)
Arg-82 → Gln (pH 8)	15. (0.27)	1.3 (0.73)	Not observed
Arg-134 → Cys	14. (0.24)	1.2 (0.76)	ND§
Arg-164 → Gln	14. (0.31)	5.7 (0.69)	2.5 (0.75)
Arg-175 → Gln	7.0 (0.84)	1.8 (0.16)	ND§
Arg-225 → Gln	8.5 (0.27)	1.2 (0.73)	1.4 (0.61)
Arg-227 → Gln	5.9 (0.18)	0.27 (0.82)	0.30 (0.83)

*Each value shown is an average of at least two independent measurements. The relative difference between the independent measurements is < 10%.

‡In parentheses are the relative amplitudes for the two M components and the slow fraction of the total absorbance change of the UV transient.

§Not determined. The signal is too noisy to analyze.

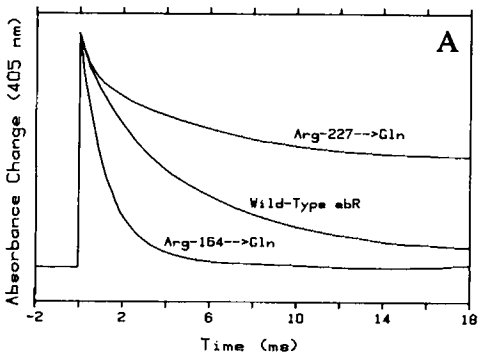
indicated, the arginine mutants studied all have similar rates which are 2–3 and 5–7 times faster than those for the fast and slow components of M formation of ebR and native PM, respectively. A similar trend is also observed for the UV transient, except for the Arg-82 and Arg-175 mutants. It is worth noting that, in general, the retinal environment is more heterogeneous in the case of the arginine mutants compared to that of ebR and native PM, as is evidenced by the increased amplitude of the fast rising component in the M formation. The populations responsible for the fast and slow components in M formation are actually reversed in the case of the Arg-82 mutant at pH 8. One might immediately suggest that this observation is similar to the effect of alkaline pH on ebR and native PM. However, it has been observed in our present and previous studies that the corresponding M decay of the Arg-82 mutant is not much different from that of ebR and native PM at pH 6 (as shown in Table 2). The observed apparent speeding up of the M formation in the Arg-82 mutant photocycle is thus a result of an increase in the relative amplitude of its fast component caused by this particular mutation. The relative amplitude of the slow-rising UV transient is slightly less in the case of the arginine mutants as compared to ebR and native PM. The correlation in the observed changes of both the formation rate constants and their relative amplitudes of the M intermediate and the UV transient suggests that both the PSB and the amino acid residue(s), whose transient gives rise to the UV transient absorption, are in the same local environment.

Comparison of the kinetic parameters of the formation of the M intermediate with that of the UV transient shows that, as in the case of ebR and native PM, the arginine mutants studied have the UV transient rise being slightly slower than that of the M formation, with the exception of the Arg-82 and Arg-175 mutants. This observation is in agreement with the previous finding for native PM (32) that formation of the UV transient is not a prerequisite for the formation of M.

The rise of the UV transient in the Arg-175 mutant is very weak to conclude whether it is due to amino acid residue or the changes in the retinal absorption. For this reason, we will not attempt to analyze it. The fact that the UV transient absorption at 296 nm is not detected for the Arg-82 mutant while its 405 nm transient for the M intermediate is strongly observed suggests the importance of Arg-82 in inducing the UV transient absorption. Recently charge perturbation of Trp-182 was shown to be responsible for the observed UV transient absorption (Wu, S., D.-J. Jang, M. A. El-Slayed, T. Marti, T. Mogi, and H. G. Khorana, manuscript submitted for publication). Because Arg-82 is charged, it could become a possible candidate for this perturbation. However, it is located below the retinal whereas Trp-182 is observed above the retinal (2). If they don't get closer during the photocycle, Arg-82 could still be indirectly responsible for this perturbation, e.g., by controlling the reaction path during the cycle such as to allow another charged species to induce the observed shift in the lowest absorption of Trp-182.

Table 2 shows the kinetic parameters of the decay of the M intermediate and UV transient in the photocycle of native PM, ebR, and the arginine mutants. Except for the Arg-164 and Arg-227 mutants, ebR as well as the

remaining mutants have transient decay kinetics of the M intermediate and the UV transient similar to those for native PM. Again, as already indicated in the case of rise kinetics, the retinal environment is less homogeneous in the arginine mutants as compared to ebR and native PM. It is interesting to observe that the apparent increased rate of the M decay is achieved in two possible ways in two different mutants. In the case of the Arg-164 mutation, the apparent M decay is increased by mainly increasing the value of the rate of the slow component while keeping the relative populations of the fast and slow components unchanged. Whereas in the case of the Arg-175 mutation the apparent M decay is enhanced by largely increasing the relative amplitude of the fast component while maintaining the relatively same value for the rate constants. Meanwhile, in the case of Arg-227 mutation, the M decay is slowed down by a factor of ~5 due to significant decrease in the value of the rate constant of the slow component (also see Fig. 1 *A* for representative traces). The UV transient also shows a similar trend, though not as dramatic (also see Fig. 1 *B* for representative traces).



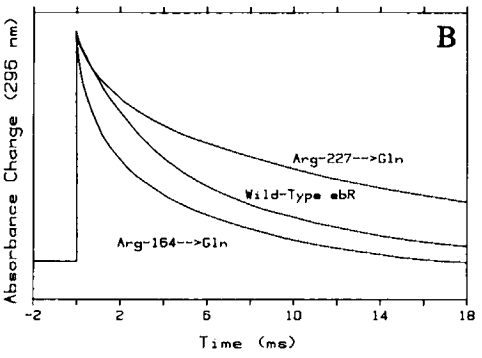


FIGURE 1 Decay kinetics of (*A*) the M intermediate monitored at 405 nm and (*B*) the UV transient monitored at 296 ± 5 nm during the photocycle of wild-type bR and mutants Arg-164 → Gln and Arg-227 → Gln. The signals shown are smoothed and normalized to have the same maximum amplitude and the unit for absorbance is arbitrary.

Previously, Packer et al. (34) reported that purple membranes treated with reagents which modify arginine and carboxylic acid residues show marked inhibition of the reprotonation phase, leading to large increase in the amount of the M species seen in the photostationary state. Their results suggest an essential role of the positively charged guanidium group of arginine and negatively charged carboxyl-containing residues of bR in the decay of the M species at the inner surface of the purple membrane, where proton uptake from the intracellular space occurs. From the recently published model of the bR structure by Henderson et al. (26), Arg-227 is located on the inner surface of the purple membrane, with Asp-96 nearby on the C helix with which it may interact. Our results then suggest that the arginine residue being modified in Packer's experiment and causing dramatic inhibition of the M decay was likely the Arg-227 residue.

Assuming that genetic modification does not induce significant spectral shift in the absorption profile of the M intermediate and the UV transient, Table 3 shows the raio of the absorption of the slow rising UV transient relative to that of the slow-rising M for the same mutant. Except for the Arg-227 mutant, ebR and the remaining arginine mutants all have ratios similar to that observed for native PM, indicating that no significant changes take place in the environment of the PSB and the amino acid residue(s) with the UV transient absorption. The fact that the Arg-227 mutant gives a ratio of one fourth the value formed for ebR suggests that replacement of Arg-227 by Gln significantly affects the formation probability of the UV transient. As mentioned above, the UV transient could possibly be a perturbed tryptophan (22) and, if so, it is Trp-182.[1] The FTIR (25) results suggest that some aspartic acids in bR could have a pK_a value

TABLE 3 **Absorption of the UV transient versus that of the slow rising M at room temperature (~22°C)***

Protein	A_{UV}/A_M^{slow}	% relative to ebR
Native bR	0.044	90
ebR	0.049	100
Arg-7 → Gln	0.040	71
Arg-82 → Gln (pH 8)	Not observed	Not observed
Arg-134 → Cys	0.040	71
Arg-164 → Gln	0.029	52
Arg-175 → Gln	0.030	61
Arg-225 → Gln	0.029	52
Arg-227 → Gln	0.015	27

*Each value shown is an average of at least two independent measurements. The relative difference between the independent measurements is < 10%.
‡The signal is too noisy to analyze.

near 9. If that is the case, then it is possible that the pH dependence of the UV transient is a result of perturbation by the ionized form of such an aspartic acid (22) or indirectly by other charges.[1] Arg-227 could either catalyze the deprotonation of this aspartic acid, being partially responsible for the charge perturbation, or indirectly control the photocycle path which involves perturbation by other charged species.

As noted earlier, the Arg-227 mutant has a much reduced value for the decay of its M intermediate. To see whether this is due to changes in the energy and/or entropy of activation, a temperature dependence study on the rate of the slow-decaying M of this mutant as well as that of ebR and native PM was carried out. The results are shown in Fig. 2. Applying the combination of both Arrhenius and Wynne-Jones-Eyring expressions for the rate constant, it was found that the values of activation energy and entropy for the decay of the M intermediate in the photocycle of native bR are E_a = 71 kJ/mol and $\Delta S^{\ddagger}$ = 29 J/K-mol, and those for ebR are E_a = 73 kJ/mol and $\Delta S^{\ddagger}$ = 29 J/K-mol. The values of these activation energies are in agreement with those previously reported (35–37). The value of the entropy of activation for native PM agrees with the positive value reported in (35) but not with the relatively smaller negative value reported in (36, 37). The values of activation energy and entropy for the decay of the M intermediate in the photocycle of the Arg-227 mutant were determined to be E_a = 16 kJ/mol and $\Delta S^{\ddagger}$ = −175 J/K-mol, both of which are much lower compared with those for native PM and ebR. Therefore, the replacement of the presumably positively charged Arg-227 by the neutral Gln has brought a substantial amount of conformational and/or environmental change, resulting in a decrease in the decay of the M intermediate. This suggests an important role of Arg-227 in the rate of the protein conformational changes leading to the reprotonation of the SB. It is now believed that Asp-96 is the internal proton donor in the reprotonation of the SB (17, 26-28). According to Henderson et al. (26), both Asp-96 and Arg-227 are present in the proton channel. Thus, it is very likely to have a direct electrostatic coupling between the two oppositely charged amino acid residues at the reprotonation stage of the bR photocycle. Disruption of this interaction by replacing the positively charged arginine with the neutral glutamine could certainly change the mechanism of the SB reprotonation to one of lower energy and entropy of activation. The observed activation energy of 16 kJ/mol for the Arg-227 mutant is near that observed for the Asp-96 mutant (38) and found for the proton diffusion in water (39). This suggests that the replacement of the charged Arg-227 could change a "structured" conformation of the protein involving Asp-96 that is used for the reprotonation of the SB to a "broken" structure which leaves only proton diffusion among water molecules (and few amino acid residues) to be the only viable means by which the SB is reprotonated.

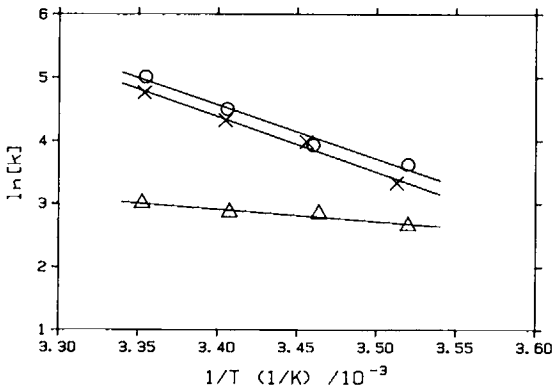


FIGURE 2 Arrhenius plots of the slow decaying M in the photocycle of native PM (○; E_a = 73 kJ/mol, $\Delta S^{\ddagger}$ = 29 J/K-mol), ebR (×; E_a = 71 kJ/mol, $\Delta S^{\ddagger}$ = 29 J/K-mol), and Arg-227 → Gln mutant (△; E_a = 16 kJ/mol, $\Delta S^{\ddagger}$ = −175 J/K-mol). The values shown in parentheses are determined based on the average of at least two independent experimental measurements which have relative differences of less than 10%.

What is not consistent with the model by Henderson et al. (26), however, are the results on the mutations at Arg-164 and Arg-175. Both of these residues are present in neither the proton channel nor the retinal pocket, yet, they show a large increase in the rates of the M decay. Obviously, these observations must be consequences of secondary effects of these substitutions on the protein structure of bR.

We would like to thank Hyun-Jin Hwang and Yue-jin Chang for installing the new system for data acquisition and analysis.

This work was supported by the Department of Energy under grant DE-FG03-88ER13828 to the University of California at Los Angeles, National Institutes of Health grant GM28289-09, and an Office of Naval Research grant N0014-82-K-0668 to Massachusetts Institute of Technology.

Received for publication 3 January 1991 and in final form 19 March 1991.

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