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The use of tryptophan mutants in identifying the 296 nm transient absorbing species during the photocycle of bacteriorhodopsin

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The transient absorption at 296 nm was part of the spectroscopic evidence that initiated the proposal that tyrosinate (Tyr^-) is formed during, and important to, the photocycle of bacteriorhodopsin (bR). Recent evidence against such a proposal comes from the results of NMR, UV Raman as well as electron cryo-microscopic structural studies. This makes it credible to assign this absorption to a charge perturbation of the lowest energy absorption of one of the tryptophan (Trp) residues in bR. The transient absorption at 296 nm is examined for each of 8 tryptophan mutants in which Trp is substituted by phenylalanine or cysteine, which absorb at shorter wavelength. It is shown that while all go through the photocycle, all but Trp-182 mutant show this transient absorption. This strongly suggests the assignment of this absorption to a charge perturbaton of the lowest energy absorption of Trp-182 during the photocycle. The chemical identity of the perturbing charge(s) is briefly discussed.

Bacteriorhodopsin photocycle; Tyrosinate formation; Tryptophan mutant; UV transient absorption

1. INTRODUCTION

Bacteriorhodopsin (bR) is a membrane protein found in the cell membrane of the halophile *Halobacterium halobium* [1–4]. It functions as a light-driven proton pump which transforms visible light into chemical energy. Upon light illumination, bR undergoes a photochemical cycle [5,6].

$$bR_{568} \xrightarrow[400\ fs]{h\nu} J_{625} \xrightarrow[5\ ns]{} K_{610} \xrightarrow[2\ \mu s]{} L_{550} \overset{H^+}{\underset{60\ \mu s}{\rightleftharpoons}} M_{412} \rightleftharpoons N_{520} \longrightarrow O_{640} \longrightarrow bR_{568}$$

The protonated Schiff base (PSB) which links the retinal to the apoprotein is deprotonated during the $L_{550} \longrightarrow M_{412}$ step, leading to the release of a proton on the extracellular side of the cell membrane, during the M decay, a proton is accepted from the cytoplasmic side, thus creating an electrochemical proton gradient which in turn is used for the metabolic process of ATP synthesis [2,5,7].

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Based on the three-dimensional structure obtained from an electron cryo-microscopy study [8], the mutagenesis studies of bR mainly carried out by Khorana's group [9–13] and many other previous results [14–16], Henderson and his co-workers [8] have proposed a possible proton pump mechanism. Several key amino acid residues that are found [8] in the so-called proton channel are involved in proton pumping, whereas residues that are present in the retinal pocket are important in the color control.

The possible involvement of tyrosine in the photocycle and the proton pumping of bR was first suggested based on the results of chemical modification [17–22]. It was proposed that a hydrogen bonding chain, involving the tyrosine residues in bR is responsible for the transfer of protons from the PSB to the membrane surface [19b,23,24]. A transient absorption was observed in the UV region (at 240 and 296 nm) on the time scale of the deprotonation of the PSB and was assigned to either the formation of tyrosinate or the charge perturbation of tryptophan [24–27]. The fact that the absorption in this region in bR decreases as the pH increases, revealing a pK_a near 10, suggested the assignment of this absorption to tyrosinate [26]. The fact that the amount and the rate of M formation is found to depend on pH, giving a pK_a value near 10, leads to the proposal that tyrosinate formation during the cycle was a prerequisite for the deprotonation of the PSB [28]. The relative amplitudes of the fast to the slow rising components of M_{412} were found to reflect the presence of an ionizable amino acid with a pK_a near 9.6 (this was

presumed to be tyrosine) that controls the environment around the PSB (thus creating heterogeneity) [29]. More recent optical, FTIR [30–33] and Raman studies [34] proposed that tyrosinate is formed during the formation of M_{412}, besides its presence in the light-adapted ground state of bR. Based on FTIR studies of bR mutants, Tyr-185 was proposed to be deprotonated in bR, protonated in K and deprotonated in M [33].

More recent observations are mounting against tyrosinate formation in bR or during M formation. The proposal that Tyr might be required for the deprotonation of the PSB was not supported by the fact that the transient assigned to Tyr^- at 296 nm, which was proposed to be a pre-requisite for the formation of M [28] actually was formed after the M formation [29]. Furthermore, mutant studies showed that none of the tyrosine replacements inhibited the proton pumping process [10]. This shed doubt that a specific hydrogen bonded chain is required for proton pumping. A combined absorption and Raman study by Maeda et al. [35] has suggested that the observed absorption at 296 nm is more likely to be the result of a charge perturbation of Trp by an aspartate group of a conjugate acid with a high pK_a which is proposed to exist in bR based on FTIR studies [36]. Recently, mutation of the most suspicious Tyr in bR, Tyr-185, showed that the Tyr-185 ⟶ Phe mutant has a transient absorption at 296 nm on the time scale of M formation [37]. This provides strong evidence against this absorption being due to Tyr^--185 during the cycle [37]. Perhaps the strongest evidence against the presence of Tyr^- in bR came from recent structural studies by Henderson et al. [8] as well as from solid state NMR studies by Griffin et al. [38] using ^{13}C tyrosines. From the structural studies, the observed distance between Tyr-185 and the PSB is too far for direct coupling [8]. The NMR results showed that only above pH 13 is Tyr^- observed in bR or in its M intermediate [38]. Recent Raman studies by Mathies et al. have confirmed this finding [39].

If the absorption at 296 nm is not due to Tyr^-, then it must be due to charge perturbation of the lowest UV absorption band of the protein which is due to Trp. The question is which Trp is indeed the one being perturbed during the cycle? In order to answer this question, we have examined the transient absorption at 296 nm for different Trp mutants in which the individual Trp residues are substituted by either phenylalanine or cysteine, which absorb so much higher in energy than Trp that its charge perturbed absorption is expected to be at shorter wavelength than 296 nm. At the same time, the absorption at 405 nm is monitored to follow the progress of the photocycle in each mutant. The results suggest that while the deprotonation of the PSB takes place in all the Trp mutants in bR, only the replacement of Trp-182 is found to eliminate the 296 nm transient absorption. This strongly suggests that it is the perturbation of the absorption of this Trp residue that leads to the transient absorption change at 296 nm.

2. MATERIALS AND METHODS

Single substitution of tryptophan residues by phenylalanine or cysteine were carried out by the cassette replacement mutagenesis [13]. The details of the cloning and expression of wild type bR (ebR) and mutants have been reported previously [13,40]. The apoproteins were regenerated with retinal and reconstituted in vehicles with the polar lipid from *H-halobium*, using a protein:lipid weight ratio of 1:1 [41].

The experimental setup for measurement of the transient absorption at 405 nm (M_{412}) and 296 nm as described previously [37] was used with some modification. A focused 0.5 ns 580 nm pulse generated from a N_2-pumped dye laser (LN 1000 and LN 102; Photochemical Research Associates Inc., London, Ont., Canada) was used as an excitation beam. The laser energy was 75 μJ with a spot of 2 mm. The 296 nm and 405 nm probe beams were the outputs of a 100-W Hg arc lamp (Pek Labs 401; Sunnyvale, CA) passed through the filters and sample and then focused into a 0.25 m monochromator (Jarrell-Ash 82-410; Waltham, MA). A photomultiplier tube (1P28A; RCA, RCA New Products Div., Lancaster, PA) was used to detect the probe light. The PMT signal was recorded by a fast response detector (Biomation 8100; Princeton Applied Research Corporation, Princeton, NJ) which was interfaced to the IBM/PC computer. Signals of 1000 or 2000 laser shots were usually averaged and then converted to transient absorption in O.D.

3. RESULTS AND DISCUSSION

The rise kinetics of the transient absorption at 296 ± 5 nm for ebR and the tryptophan mutants are shown in Fig. 1. The observed absorption changes (ΔA) have been normalized to the same concentration for all the mutant samples according to their individual static absorption spectra. The kinetics of ebR and most mutants clearly show both the fast- (shown by solid vertical line) and the slow-rising (shown by dots) components. A great deal of work is carried out in order to assign the two components observed on similar time scale in bR. The spectrum obtained for the absorption changes of the fast component is found [27] to match the difference spectrum of the light- and dark-adapted bR. The latter gives the changes in the spectrum when retinal isomerizes from all-*trans* to 13-*cis*. Thus, the fast component is assigned to the retinal absorption changes resulting from the isomerization of the retinal chromophore during the cycle [27]. Using ultrafast spectroscopy, this process is found to occur during the cycle in 400 fs by both optical [42] and Raman [43] techniques. The slow component occurs on the same time scale as the deprotonation process. Both are found to have similar activation energies [29] which is similar to that observed for the fluorescence quenching process of the Trp fluorescence during the cycle [44]. This together with the fact that its amplitude changes with pH showing a pK_a value near 10 [26] strongly suggested that it is due to changes in the absorption of protein molecules with maxima not far from the wavelength of Tyr or Trp.

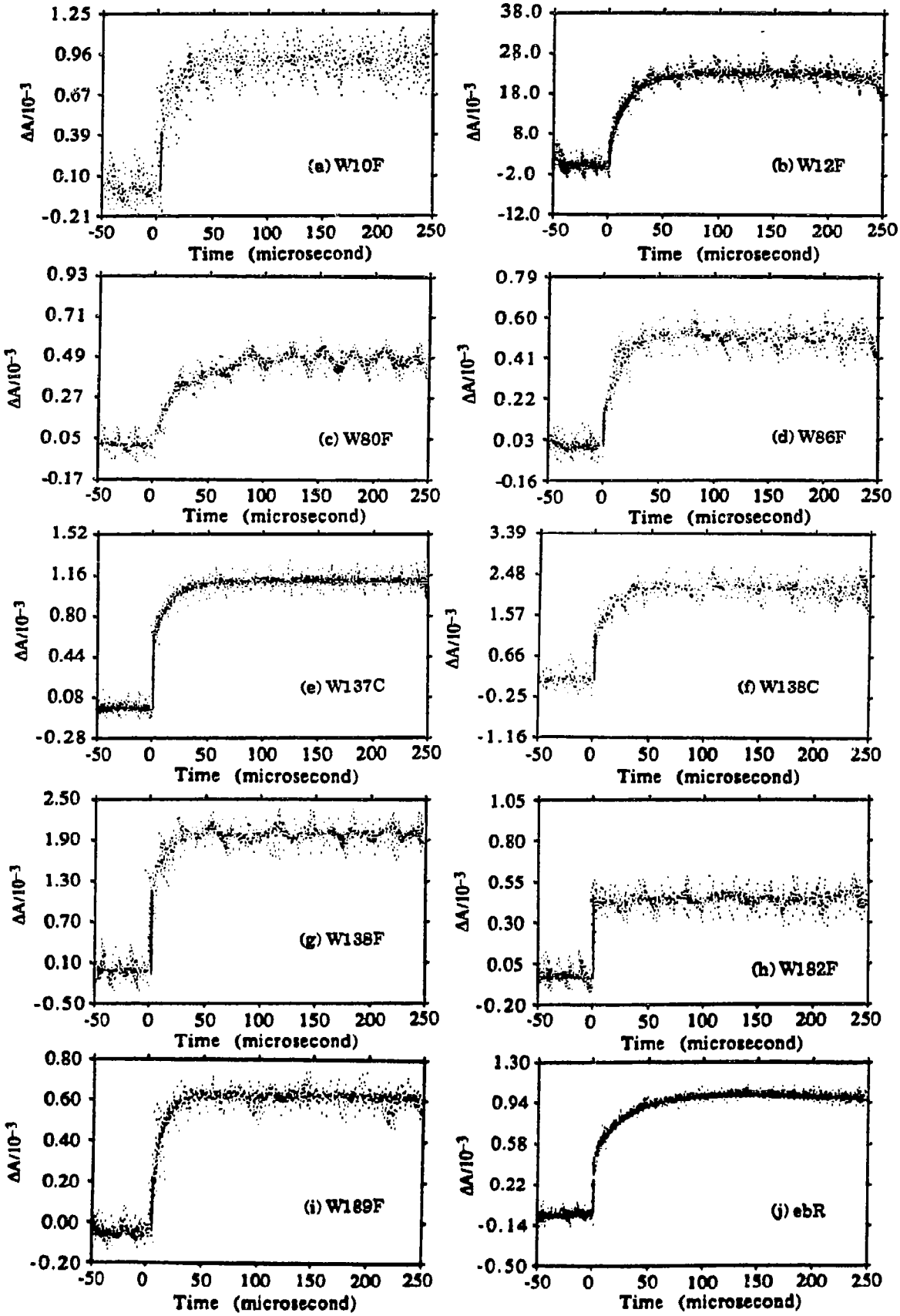

Fig. 1. Formation kinetics of transient absorption at 296 ± 5 nm for ebR and the tryptophan mutants at pH 6. Samples were photolyzed at 580 nm. Solid lines indicate the absorption change due to the retinal isomerization and the dots show the ones due to the protein absorption. Observed transient absorption (ΔA) is normalized to the same concentration for all the mutant samples using static chromophore absorption at 568 nm.

The lack of observation of the fast-rising component for some mutants (e.g. W80F and probably W12F in Fig. 1) suggests that the retinal absorption at 296 nm does not greatly change on going from bR_{568} to M_{412} in these mutants. The most important result shown in Fig. 1 is that only the fast-rising component and not the slow one is observed for the W182F mutant (Fig. 1h), which means that no transient absorption change of the protein is observed for this mutant. It is known that if M formation is inhibited, e.g. by removal of metal cations, the 296 nm transient is not formed [45]. For this reason we have examined the M formation kinetics for the Trp-182 ⟶ Phe mutant. Fig. 2 shows its rise kinetics compared with that of ebR. From this, it is clear that M is formed during the cycle of the W182F mutant although at a slower rate than in ebR. Thus, while M is formed, the 296 nm transient is not observed when Trp-182 is replaced by an amino acid (phenylalanine) whose absorption is located at a much higher frequency. These results provide strong evidence that Trp-182 is the tryptophan residue whose absorption is either perturbed or alternatively whose substitution changes the protein structure to prevent another Trp from having its absorption perturbed during the photocycle. The latter possibility can be eliminated since, if it were true, one would expect that the replacement of another Trp (the one whose absorption gets perturbed during the cycle) should also eliminate the slow transient absorption component at 296 nm. Since this is not observed, one is led to the conclusion that the 296 nm transient absorption results from charge perturbation of the Trp-182 absorption during the photocycle.

Next, let us discuss the possible candidates for the charged species that could perturb Trp-182 during the M_{412} formation. The observed shift in the absorption frequency $\Delta\nu$ of a molecule like Trp due to an electrical field $\vec{E}$ resulting from a nearby charge is given by [46,47];

$$\Delta\nu = 1/h(\vec{E}\Delta\vec{\mu})$$

where $\Delta\vec{\mu}$ is the change in the dipole moment of the molecule upon excitation. From this equation, shifts can occur to higher (blue shift) or to lower (red shift) frequencies depending on the sign of the charge (positive or negative sign of $\vec{E}$) and its relative orientation with respect to the molecule whose absorption is being monitored. From the above, it is clear that either positive or negative charges can give rise to the observed red shift of Trp-182 in bR.

The intensity of the transient absorption at 296 nm is found to decrease with increasing pH and seems to be controlled by an acid-base equilibrium involving an acidic group with a pK_a value between 9 and 10 [26,28,29]. Maeda et al. [35] suggested that since some aspartic acids are found to have pK_a values as high as 9 [36] the 296 nm absorption could result from perturbing Trp absorption by an aspartate residue. Asp-115 and

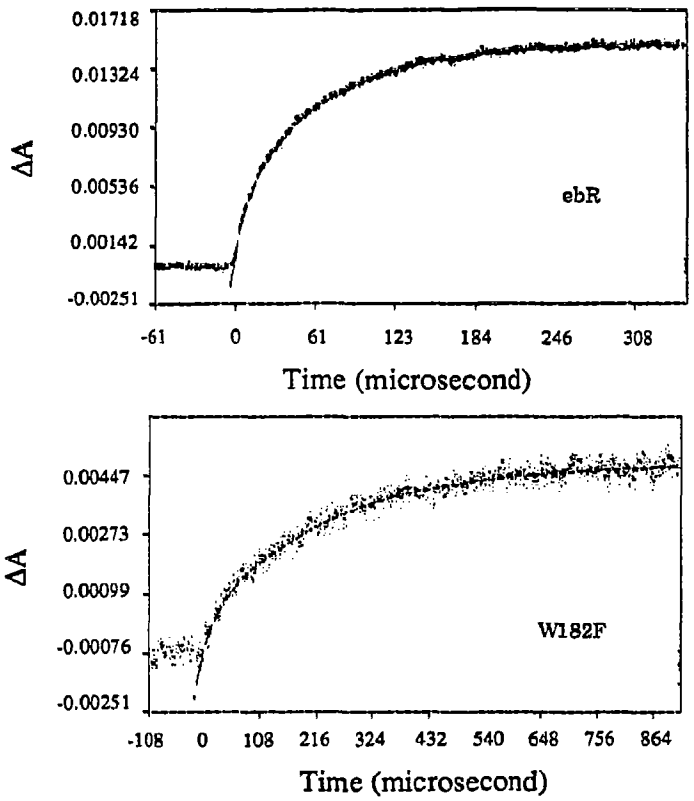

Fig. 2. M_{412} formation kinetics of transient absorption at 405 nm for ebR and W182F mutant at pH 6. Samples were photolyzed at 580 nm.

-96 are the ones that are protonated in bR_{568} [8]. It is known that Asp-96 deprotonates as it donates its proton to the Schiff base during M decay [8]. This occurs on the millisecond time scale and thus Asp-96 can not be the aspartate that perturbs the Trp to give the 296 nm absorption (which appears in tens of microseconds). This leaves Asp-115 as a possible candidate responsible for the pH dependence of the transient absorption at 296 nm. From the proposed model of the cycle [36], there is an aspartic acid (Asp(1) which is Asp-115), that deprotonates during L-formation (2 μs). However, this aspartate residue reprotonates during M formation [36]. This eliminates it as a candidate that directly perturbs Trp-182. It does not, however, eliminate it as the carboxylic acid whose deprotonation in K ⟶ L and reprotonation in L ⟶ M allows for another positively or negatively charged species to perturb the absorption of Trp-182 during the L ⟶ M step. According to Henderson's model [8], two negative charges (Asp^--85 and Asp^--212) and two positive charges (Arg-82 and PSB) are present in the retinal pocket. Both the PSB and at least one (Asp-85) of the two aspartates (or all according to the FTIR studies [36]) cannot be the candidates, as they lose their charge during the appearance of the transient absorption at 296 nm. Recently, a metal cation is proposed to be also present within the retinal pocket [48,49]. The removal of metal cations

is found to eliminate both the M_{412} formation as well as the 296 nm absorption [45]. From this observation alone one cannot conclude whether metal cation if one is indeed present in the retinal pocket perturbs the Trp-182 absorption during the cycle, since the latter perturbation occurs in the protein conformation of the M_{412} intermediate, which is not formed upon removal of metal cations. Recently, it was found that the genetic substitution of Arg-82 by glutamine increases the rate of M_{412} formation but eliminates the 296 nm transient absorption [50]. Unfortunately, Arg-82 is far away from Trp-182 according to Henderson's structure [8]. It could, however, be important in controlling the type of the protein conformation involved in forming M which allows another charge to perturb Trp-182 absorption. It is thus difficult at this time to conclude which of the charged species in bR perturbs the Trp-182 during the M formation. It might very well be a charged species which is not in the active site but rather within the transmembrane region and its charge could be felt by Trp-182 during the photocycle, e.g. R175.

It should also be noted that normalized amplitude (ΔA) of transient absorption for W12F mutant is about 20 times greater than that observed in ebR as shown in Fig. 1b and Fig. 1j, but the amount of M formation is about the same as in ebR (our unpublished results). We have checked that this observation is not due to the experimental artifact. This means that the replacement of Trp-12 must change the protein conformation in such a way not to affect the cycle photo-efficiency but greatly increase the transient absorption at 296 nm. The latter could be a result of increasing the number of the other Trp residues being perturbed as well as greatly increasing the extinction coefficient of the absorption of perturbed Trp residue(s) during the cycle.

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