et al., 1981). How nonglycosylated TPA will react as an antigen, its biological half-life, and its interaction with extracellular inhibitors await further study.

Acknowledgments

The expert technical assistance of Jenna Walls is gratefully acknowledged.

Registry No. Plasminogen, 9001-91-6.

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Selective Nitration of Tyrosines-26 and -64 in Bacteriorhodopsin with Tetranitromethane†

Peter Scherrer* and Walther Stoeckenius

ABSTRACT: Nitration of tyrosine-26 at pH 9.0 in bacteriorhodopsin does not change its absorption spectrum but lowers the apparent p*K* of the alkaline transition to a blue-shifted chromophore from about pH 12.0 to 10.6. This effect is reversed by reducing the nitrotyrosine-26 to aminotyrosine which demonstrates that the protonation state of tyrosine-26 and the alkaline chromophore transition are correlated. Nitration of tyrosine-64 resulted in a shift of the purple complex from 570 to 535 nm at neutral pH. The alkaline transition p*K* of such a nitrated membrane was below 10 but was clearly independent of the protonation state of tyrosine-64 because it is not reversed by reduction of the nitrotyrosine. Nitrotyrosine-26 showed spectral properties similar to L-nitrotyrosine in aqueous environment while nitrotyrosine-64 showed only a 360-nm absorbance in the apomembrane but not in the retinal-containing membrane. Both tyrosines are accessible to water-soluble reagents.

Bacteriorhodopsin (bR),[1] the only protein in the purple membrane of *Halobacterium halobium*, functions as a light-driven proton pump [for a review, see Stoeckenius et al. (1979) and Stoeckenius & Bogomolni (1982)]. The bR molecules are arranged within the membrane in a rigid hexagonal lattice, and structural analysis by electron microscopy suggests a three-dimensional structure with seven helices spanning the membrane (Unwin & Henderson, 1975). The primary sequence is known (Ovchinnikov et al., 1979; Khorana

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[1] Abbreviations: bR, bacteriorhodopsin; bO, bacterioopsin; pm, purple membrane; TNM, tetranitromethane; N-Tyr, nitrotyrosine; bR-TNM-d, bR modified with TNM in the dark; bR-TNM-l, bR modified with TNM in the light; HPLC, high-pressure liquid chromatography; CT, chymotrypsin; CNBr, cyanogen bromide; TFA, trifluoroacetic acid; PTH, phenylthiohydantoin; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; SDS, sodium dodecyl sulfate.

0006-2960/84/0423-6195$01.50/0

et al., 1979), and several detailed models of the structure have been proposed (Ovchinnikov et al., 1979; Khorana et al., 1979; Engelman et al., 1980; Agard & Stroud, 1982). Like visual pigments, bR contains an ϵ-retinylidene chromophore (Oesterhelt & Stoeckenius, 1971), and the retinal–Schiff base linkage to lysine-216 (Katre et al., 1981; Bayley et al., 1981; Lemke & Oesterhelt, 1981b; Mullen et al., 1981) is protonated (Lewis et al., 1974). The position of its main absorption band at 558 nm in the dark and 568 nm in the light is explained by additional noncovalent protein–retinal interactions (Blatz et al., 1972; Nakanishi et al., 1980). The chromophore undergoes a cyclic photoreaction comprising at least four spectroscopically distinct intermediates (Lozier et al., 1975; Lozier & Niederberger, 1977), a transient deprotonation of the Schiff base (Lewis et al., 1974), and a transient all-trans to 13-cis isomerization of the retinal (Pettei et al., 1977). The molecular mechanism of the light-induced proton translocation is not known.

Modification of tyrosine residues in bR affects the absorption spectrum and photoreaction kinetics of the chromophore (Scherrer et al., 1981; Lemke & Oesterhelt, 1981a; Lemke et al., 1982), but, so far, no specific modification of a single group in an identified position of the peptide chain has been described; rather, the effects of specific groups on the protein structure and function have been deduced indirectly. Here we report selective nitration of tyrosine residues with modified reactivity due to their environment.

Materials and Methods

Materials. Purple membrane (pm) from *H. halobium* (strain ET1001 provided by Dr. H. J. Weber) was isolated as described (Oesterhelt & Stoeckenius, 1974). The pm suspension was stored either at 4 °C in 2 M NaCl or at −80 °C in 40% aqueous sucrose solution. Concentrations of bR were determined spectroscopically by using $\epsilon_{570} = 63\,000\ M^{-1}\ cm^{-1}$ and $M_r = 26\,000$. 3-Nitrotyrosine, 3-aminotyrosine, hydroxylamine hydrochloride, chymotrypsin (CT) type VII from bovine pancreas, and tetranitromethane (TNM) were obtained from Sigma. Formic acid 95–97%, obtained from Aldrich, was distilled prior to use. Cyanogen bromide (CNBr) was from Eastman Organic Chemicals, trifluoroacetic acid (TFA) was from Pierce, ethanol 200 proof was from Gold Shield Chemical, and Sephadex LH-20 and Sephadex LH-60 were from Pharmacia Fine Chemicals.

Nitration of Purple Membrane with TNM. (*a*) *Nitration in the Dark* (*bR-TNM-d*). To 10 mL of pm suspension (0.46 μmol) in 50 mM Tris-HCl buffer, pH 9.0, gassed with nitrogen, was added 46 μL of 0.1 M TNM in ethanol to give a 10-fold molar excess over bR. The sample was kept in the dark at room temperature and the reaction stopped after 2 h by centrifuging (100000*g*, 20 min) and washing the membranes 4 times in distilled H_2O.

(*b*) *Nitration in the Light* (*bR-TNM-l*). The same protocol as for bR-TNM-d was used except that pm was suspended in 50 mM sodium phosphate buffer, pH 6.0, placed in ice water, and illuminated with yellow light (Corning 3-69 filter) from a projector lamp (250 W) throughout the reaction.

Reduction of Nitrated Purple Membrane. Twelve milligrams of nitrated pm (~5 μmol) was resuspended in 6 mL of 50 mM Tris-HCl buffer, pH 8.2, reduced with 100 μL of freshly prepared 1 M $Na_2S_2O_4$ (100 μmol of sodium dithionite) for 30 min at room temperature, and then washed 4 times in distilled H_2O by centrifugation.

Absorption spectra of native and nitrated pm were recorded with an Aminco DW-2a spectrophotometer linked to a Nicolet 1074 data acquisition system.

Identification of Modified Groups by Peptide Mapping and Sequencing. Three types of samples were prepared for peptide mapping: (a) pm and modified pm were bleached with 400 mM hydroxylamine, pH 7.0, with light (Becher & Cassim, 1977), for 3 h and washed 4 times with distilled H_2O by centrifugation. The apomembrane was resuspended in 50 mM Tris-HCl, pH 8.0, and 5 mM $CaCl_2$ to give 2 mg/mL protein and cleaved with chymotrypsin (0.02 mg of CT/mg of protein) for 5 h at 37 °C (Gerber et al., 1977). The membrane was washed 3 times with distilled H_2O by centrifugation and the pellet solubilized as in (c). (b) The pm preparations were suspended in 50 mM Tris-HCl, pH 8.0, and 100 mM $CaCl_2$ and cleaved with chymotrypsin as described for the apomembrane above. (c) Centrifuged pellets of the pm preparations without prior CT cleavage were solubilized in 1 mL of 97% formic acid in a bath sonicator (Laboratory Supplies Co., Inc.), and 2.8 mL of ethanol was added.

The samples were delipidated, and in cases a and b, the chymotrypsin fragments (CT-I, amino acids 72–248; CT-II, amino acids 1–71) were separated by passing them through a Sephadex LH-60 column (26 × 540 mm, 0.4 mL/min, 3 mL/fraction) with 95% HCOOH/ethanol (1:2.8) as solvent (Gerber et al., 1979). The protein fractions were pooled, and the solvent was removed under vacuum in a rotary evaporator at 40 °C.

CNBr Cleavage of bR, CT-I, and CT-II. The samples (0.4 μmol) were dissolved in 2 mL of 95% HCOOH and diluted with 0.7 mL of distilled water to 70% HCOOH. A 50-fold molar excess of CNBr over methionine was added, and the samples were kept in the dark at room temperature for 24 h (Gerber & Khorana, 1982). The excess reagents and solvent were then removed under vacuum in a rotary evaporator and the CNBr fragments separated by chromatography on Sephadex LH-60 and LH-20 and by HPLC.

Gel Permeation Chromatography. The samples were dissolved in 95% HCOOH, and ethanol was added to give a ratio of 1:2.8 (HCOOH:ethanol). They were applied to columns of Sephadex LH-20 (9 × 550 mm) and LH-60 (26 × 540 mm for samples ≥0.2 μmol of protein, 10 × 500 mm for <0.2 μmol of protein) preequilibrated with 95% HCOOH/ethanol (1:2.8). The columns were eluted with 95% HCOOH/ethanol (1:2.8) at a 15 mL/h flow rate and 3 mL/sample for the 26 × 540 mm LH-60 column. For the 10 × 500 mm LH-60 column, the flow rate was 3 mL/h, and samples of 0.4 mL were collected. The flow rate for the LH-20 column was 2 mL/h with 0.25 mL/sample.

Reverse-Phase HPLC of Peptide Fragments. The peptides were dissolved in 95% HCOOH and 20-nmol samples (400 nmol/mL) injected into a 0.39 × 30 cm μBondapak C_{18}/10-μm column (Waters) equilibrated with 90% solvent A (5% HCOOH in water) and 10% solvent B (5% HCOOH in ethanol) and eluted with a linear gradient as indicated in the figures. The flow rate was 1 mL/min. In some cases, 0.1% trifluoroacetic acid (TFA) was used instead of 5% HCOOH in both solvents.

SDS electrophoresis was carried out according to Laemmli (1970) in 12% and 16% polyacrylamide slab gels, and the gels were stained with Coomassie brilliant blue.

Amino Acid Analysis. The protein samples were hydrolyzed under vacuum in 6 N HCl for 20 h at 110 °C. Analysis was carried out on a Beckman 121M amino acid analyzer. Nitrotyrosine elutes 12 min after phenylalanine, and this peak was used to calculate the amount of nitrotyrosine in nitrated pm. For analysis of tryptophan, the samples were hydrolyzed in 4 N methanesulfonic acid.

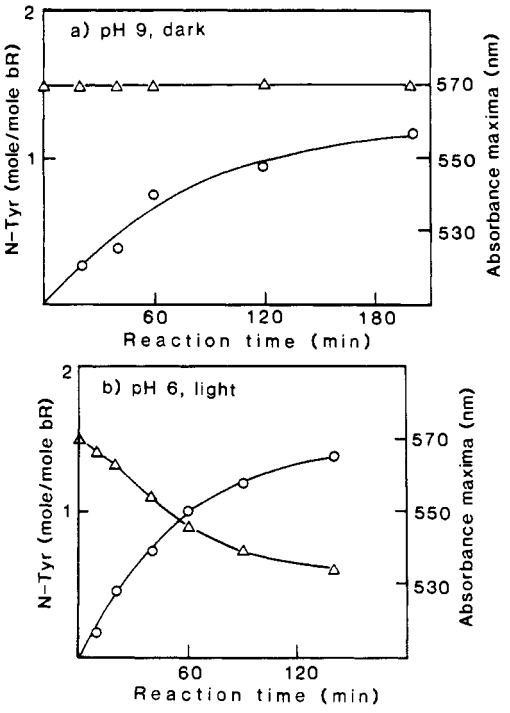

FIGURE 1: Change of chromophore absorbance in bacteriorhodopsin upon nitration. (a) 46 μL of 0.1 M TNM in ethanol was added to 0.46 μmol of pm in 10 mL of 50 mM Tris-HCl buffer, pH 9.0. Aliquots were mixed with 10% aqueous mercaptoethanol and washed with water. The absorption maxima (△) were taken from spectra measured at pH 7.0 after light adaptation. N-Tyr (○) was determined by spectrophotometric titration at 430 nm. (b) Same as (a), except pm was suspended in 50 mM phosphate buffer, pH 6.0, and illuminated.

Automated Edman Sequencing. Peptides were dissolved in 0.5 mL of 20% formic acid and applied together with 2 mg of polybrene (Sigma) to the cup of a Beckman 890C sequencer. The first cycle was double coupled; for the following, a single-coupling, single-cleavage 0.1 M Quadrol program (122974) was used. After conversion in 1 N HCl at 80 °C for 10 min, PTH-amino acids were identified by high-performance liquid chromatography as described (Weisgraber et al., 1981). PTH-nitrotyrosine elutes right before PTH-tryptophan, very well separated from PTH-tyrosine as characterized with standards.

Results

Modification of bR with TNM. Dark-adapted bR in pm reacted with TNM at alkaline pH in the dark, leading to the formation of nitrotyrosine (N-Tyr) as determined by spectrophotometric titration (Riordan et al., 1967). After 2 h, about 1 mol of N-Tyr per mole of bR was formed (Figure 1a), and the absorption of the chromophore remained unchanged. The formation of N-Tyr then continued at a much slower rate, yielding about 2 mol of N-Tyr/mol of bR after 24 h still without detectable change in the chromophore absorption. Illumination of this nitrated pm shifted the absorption maximum to 570 nm with a 13% increase in absorbance as in unmodified bR.

TNM reacts with the tyrosyl ion, and, as expected, when pm was exposed to TNM at pH 6.0 in the dark at 0 °C or at room temperature, no detectable N-Tyr was formed. However, when the same reaction mixture was exposed to light, N-Tyr was formed, and the absorption maximum of the bR chromophore shifted to 535 nm. The change of the chromophore absorbance closely followed the N-Tyr formation and reached a steady state in 1.5–2 h (Figure 1b). At this point, the amino acid analysis showed only 0.7 mol of N-Tyr/mol of bR. (The spectrophotometric titrations give too high values because the alkaline form of bR absorbs in the same region, as will be discussed later.) We calculated an extinction coefficient of 46 000 ± 1000 at 535 nm for bR modified in the light with TNM, which is similar to the value obtained by Lemke and Oesterhelt for more extensively nitrated pm (Lemke & Oesterhelt, 1981a). We also monitored the near-UV absorbance during the reaction and found no significant absorbance change. A loss of absorbance would have indicated the modification of tryptophan (Cuatrecasas et al., 1968).

Identification of the N-Tyr Position in the Sequence of bR. The cleavage of bR with chymotrypsin (CT) by the method of Gerber et al. (1979) requires bleaching with hydroxylamine. Hydroxylamine is a reducing agent, and 70% of 3-nitro-L-tyrosine (130 μM) was reduced in a 1 M hydroxylamine solution within 14 h; we therefore attempted to omit the bleaching and found that in 100 mM $CaCl_2$ CT cleaves over 60% of native bR within 5 h as judged by SDS gel electrophoresis. The bR-TNM-d preparation showed a similar CT cleavage as native bR in 10 mM $CaCl_2$ but was completely cleaved in 100 mM $CaCl_2$. The bR-TNM-l preparation was cleaved completely even at the low $CaCl_2$ concentration. The CT-cleaved membranes were solubilized in 95% formic acid and CT-I (amino acids 72–248) and CT-II (amino acids 1–71) separated by chromatography on Sephadex LH-60 (see Materials and Methods). These acidic conditions stabilize the retinal–Schiff base linkage, and the retinylidene peptide is detected by its 360-nm absorbance; it elutes with CT-I. CT-II of bR-TNM-d and bR-TNM-l also showed absorbance at 360 nm while no absorbance was seen in CT-II of native bR at that wavelength. Upon alkalinization, this absorbance shifts from 360 to 430 nm, which is characteristic of nitrotyrosine, but no such shift occurs in CT-I preparations. We conclude that nitrotyrosine is present in CT-II only. The CT-I and CT-II peptides were further cleaved with CNBr and the resulting peptides separated by HPLC on a C_{18} reverse-phase column. The CT-I peptides from bR-TNM-d showed a chromatogram similar to that of unmodified bR without any additional 360-nm absorbance, while the same peptides from bR-TNM-l showed an increase in 360-nm absorbance for peptides 4 and 5 (Figure 2). The chromatograms of CT-II (Figure 3) showed one major yellow peptide each, apparently peptide 4 for bR-TNM-d and peptide 3 for bR-TNM-l. The N-Tyr-containing peptides did elute with a different retention time than the unmodified peptide. The total increase in 360-nm absorbance for the other peptides was less than 10%.

The yellow peptides were collected and subjected to analysis by automated Edman degradation. Peptide 3 from CT-II of bR-TNM-l showed the sequence Leu-Leu-Gly-N-Tyr-Gly-... with only N-Tyr at step 4. This sequence identifies it as Tyr-64. PTH-nitrotyrosine elutes right before PTH-tryptophan characterized by standards. Since there was a 360-nm absorbance increase for the CNBr peptides 4 and 5 from CT-I, these peptides were also sequenced. The amount of N-Tyr found in these peptides was less than 10% of the total tyrosine recovered in the same step.

Peptide 4 from bR-TNM-d showed the sequence Gly-Leu-Gly-Thr-Leu-N-Tyr/Tyr-... with about 80% N-Tyr in step 6. This is the sequence of peptide 21–32 with Tyr in position 26. Peptide 4 appears as a double peak but represents one CNBr fragment due to homoserine–homoserine lactone equilibrium as confirmed by end-group analysis. In addition, traces of N-Tyr were found in the first degradation step,

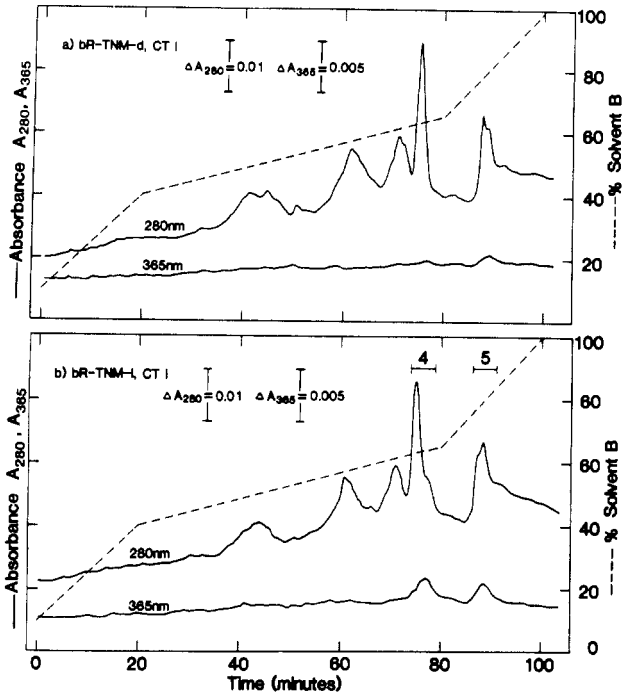

FIGURE 2: HPLC of CNBr-cleaved chymotrypsin fragment CT-I (amino acids 72–248) of (a) bR-TNM-d and (b) bR-TNM-l. For experimental conditions, see Figure 3.

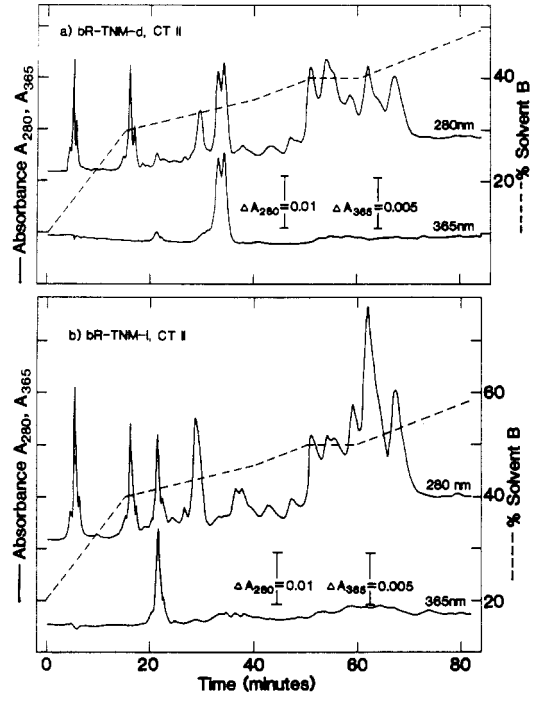

FIGURE 3: HPLC of CNBr-cleaved chymotryptic fragment CT-II (amino acids 1–71) of (a) bR-TNM-d and (b) bR-TNM-l. The peptides were dissolved in 95% HCOOH, and 50 μL containing 10–20 nmol was injected into a μBondapak C_{18} (0.39 × 30 cm) column. The column had been preequilibrated with 90% solvent A (5% HCOOH in H_2O) and 10% solvent B (5% HCOOH in ethanol) and eluted at 1 mL/min with a gradient as indicated. The absorbance was monitored at 280 and 365 nm.

indicating that Tyr-57 was nitrated to some extent since peptide 57–60 is the only CNBr peptide starting with Tyr.

We conclude that in bR-TNM-l Tyr-64 and in bR-TNM-d Tyr-26 are effectively nitrated with high selectivity. When the nitrated pm preparations were treated with sodium dithionite to reduce the exposed N-Tyr, both peptide 3 of bR-TNM-l and peptide 4 of bR-TNM-d lost their absorbance at

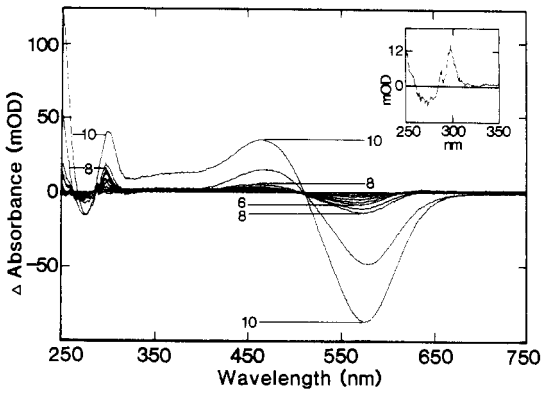

FIGURE 4: pH titration of purple membrane. A dark-adapted pm suspension (50–100 μg/mL) in 20 mM NaCl was titrated with aliquots of 1 or 5 M NaOH to the pH indicated. The spectra were recorded, and the difference to the spectra at pH 6.4 was plotted for pH (1) 7.5, (2) 8.7, (3) 9.2, (4) 9.7, (5) 10.2, (6) 10.8, (7) 11.2, (8) 11.65, (9) 12.0, and (10) 12.2. Insert: Difference spectra from 250 to 350 nm for pH 11.65 − pH 7.0.

360 nm and eluted with a different retention time. Therefore, N-Tyr-26 and -64 are accessible to water-soluble reducing agents.

Spectral Properties of the Nitrated Membranes. Addition of alkali to pm leads to a blue shift of the bR absorbance, which is caused by depletion of the 570-nm chromophore and the appearance of a species with an absorption maximum at 460–470 nm (Muccio & Cassim, 1979) (Figure 4). The transition has an isosbestic point at 520 nm. The extinction coefficient for the 580-nm chromophore depletion is about twice as large as for the corresponding 465-nm increase. Simultaneous with formation of the 470-nm species, the absorbance increases at 300 nm and decreases at 275 nm. The absorbance increase at 300 nm is characteristic for deprotonation of tyrosine (Edelhoch, 1962). However, the depletion at 275 nm is larger than expected for the tyrosine deprotonation estimated from the 300-nm absorbance increase, and the residual absorbance decrease shows a structure (Figure 4, insert) similar to tryptophan perturbation caused by a negative charge (Donovan, 1969). This alkaline transition in native pm shows a pK of about 12 and, therefore, may be caused by tyrosine deprotonation. Since nitrotyrosine has a considerably lower pK than tyrosine, we titrated the alkaline transition in the nitrated membranes.

Addition of alkali to bR-TNM-d also causes a depletion at 580 nm with an isosbestic point at 515 nm (Figure 5a). However, the corresponding absorbance increase in the blue with λ_{max} between 440 and 450 nm is larger, and its spectrum is broader than in native pm. In addition, another isosbestic point at 380 nm and a depletion at 360 nm are observed. This depletion is typical of nitrotyrosine, which is known to have a transition from the undissociated form absorbing at 360 nm (ϵ = 2200 M^{-1} cm^{-1}) to the ionized form with λ_{max} = 428 nm (ϵ = 4100 M^{-1} cm^{-1}) with an isosbestic point at 380 nm (Sokolovsky et al., 1966). The broader and larger absorbance band in the blue is most likely due to two chromophores, the nitrophenolate anion and the alkaline retinal chromophore. The decreases at 580 and 360 nm and the increase at 440 nm are synchronous and have an apparent pK of 10.6, 1.5 units lower than in native pm. This strongly suggests that deprotonation of nitrotyrosine-26 correlates with and may cause formation of the alkaline chromophore. This conclusion is confirmed by the following observation.

3-Nitrotyrosine can be reduced with sodium dithionite ($Na_2S_2O_4$) to 3-aminotyrosine (Sokolovsky et al., 1967), which

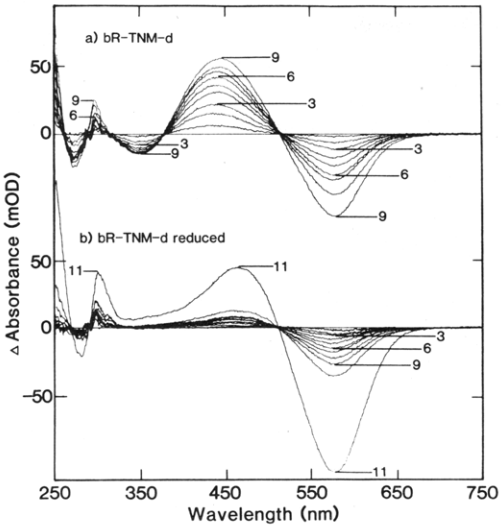

FIGURE 5: pH titration of bR-TNM-d (a) nitrated pm and (b) nitrated and subsequently reduced pm suspension under conditions as in Figure 4. The difference to the pH 7.4 spectra was plotted for pH (1) 8.4, (2) 9.05, (3) 9.5, (4) 10.0, (5) 10.4, (6) 10.85, (7) 11.25, (8) 11.65, and (9) 11.9 in (a) and the difference to pH 6.6 for pH (1) 7.5, (2) 8.5, (3) 8.95, (4) 9.45, (5) 9.85, (6) 10.2, (7) 10.6, (8) 11.0, (9) 11.4, (10) 11.85, and (11) 12.2 in (b).

increases the pK of the phenolic hydroxyl group to the value of unmodified Tyr. 3-Aminotyrosine has no absorption bands at 360 and 430 nm but exhibits a maximum at 288 nm at neutral pH and 302 nm at high pH. bR-TNM-d was treated with sodium dithionite and the reduced membrane titrated. The resulting spectral changes (Figure 5b) in the visible are virtually indistinguishable from those of unmodified membranes with a pK of about 12 for the alkaline transition to the blue species with λ_{max} = 465 nm. A comparison of the nitrated and reduced membrane titrations clearly shows the contribution of nitrotyrosine to the absorbance increase at 440 nm. Note that the reduced membrane shows twice the absorbance change at 300 nm of the nitrated membrane, because the extinction coefficient of aminotryosine is about twice that of nitrotyrosine at this wavelength. In both membranes, nitrated and nitrated–reduced, an absorbance decrease also occurs at 275 nm in the alkaline transition. It is larger than expected for nitro- or aminotyrosine and shows some features of tryptophan perturbation as described earlier for native membrane.

The alkaline titration of bR-TNM-l containing mainly N-Tyr-64 shows a depletion maximum at about 550 nm and a simultaneous absorbance increase at 440 nm with an isosbestic point at 495 nm (Figure 6a). The pK for the transition is about 10. Note that there is little, if any, absorbance decrease at 360 nm. After reduction with sodium dithionite, the isosbestic point remains unchanged at 495 nm, and so does the depletion at 545 nm while there is considerably less absorbance increase in the blue and its λ_{max} is red shifted to 450 nm (Figure 6b). The difference in the spectra in Figure 6a,b can be explained as the contribution of nitrotyrosine to the blue absorbance. With ϵ_{430nm} = 4100 M^{-1} cm^{-1} for nitrotyrosine, this change corresponds to 0.8 mol of nitrotyrosine per mol of bR. The reduction of nitrotyrosine-64 did not change the pK of the alkaline transition significantly; it remained near 10.

In bR-TNM-l we would expect an absorbance decrease at 360 nm upon alkalinization as the 430-nm absorbing nitrophenolate anion is formed. Its greatly diminished amplitude at 360 nm suggests that Tyr-64 interacts with another group (Lemke & Oesterhelt, 1981a), e.g., the retinylidene moiety. To test this possibility, the retinal of nitrated bR was removed

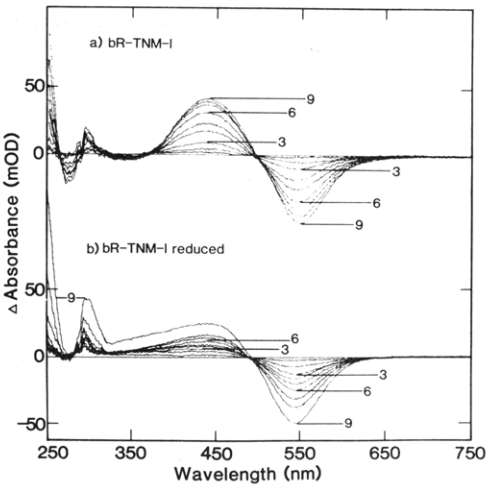

FIGURE 6: pH titration of bR-TNM-l (a) nitrated pm and (b) nitrated and subsequently reduced pm suspension under conditions as in Figure 4. The difference to the pH 6.6 spectra was plotted for pH (1) 7.5, (2) 8.1, (3) 8.8, (4) 9.5, (5) 9.9, (6) 10.5, (7) 11.0, (8) 11.5, and (9) 11.9 in (a) and the difference to pH 7.2 spectra for pH (1) 8.1, (2) 8.8, (3) 9.45, (4) 9.8, (5) 10.25, (6) 10.8, (7) 11.3, (8) 11.8, and (9) 12.2 in (b).

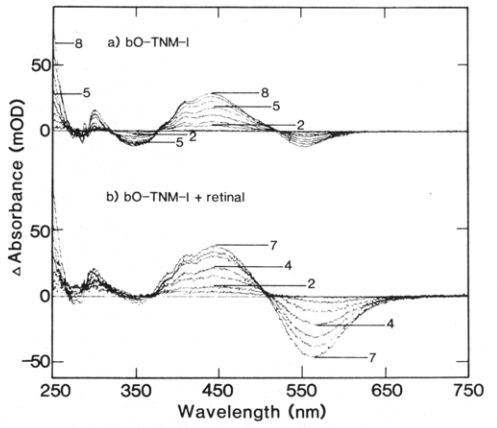

FIGURE 7: pH titration of (a) apomembrane from bR-TNM-l and (b) after subsequent reconstitution with *all-trans*-retinal followed by dark adaptation as in Figure 4. The difference to the pH 6.5 spectra was plotted for pH (1) 7.2, (2) 8.3, (3) 8.9, (4) 9.5, (5) 10.1, (6) 10.7, (7) 11.2, and (8) 11.6 in (a) and the difference to pH 7.0 spectra for pH (1) 7.5, (2) 8.1, (3) 8.8, (4) 9.5, (5) 10.3, (6) 10.9, and (7) 11.5 in (b).

by illumination in 0.4 M hydroxylamine, pH 7.0, and the resulting apomembrane titrated. Both bR-TNM-d and bR-TNM-l do bleach within 1 h in the light but are completely stable over 24 h in the dark. Within 1 h under these conditions, hydroxylamine did not cause much reduction of the nitrotyrosine as judged by peptide analysis with HPLC. The spectral changes upon titration of bR-TNM-l apomembrane are shown in Figure 7a. (The bR-TNM-l used for the titration studies was only about 80% bleached.) The spectral changes are now those expected for nitrotyrosine with an increase at 430 nm, a simultaneous depletion at 360 nm, and the isosbestic point at 380 nm. The pK is at 9.5. When *all-trans*-retinal was added to the bleached bR-TNM-l, a more than 25-nm blue-shifted chromophore was regenerated (λ_{max} = 540–545 nm). The titration of this reconstituted membrane (Figure 7b) shows a greatly reduced absorbance change at 360 nm. Overall, the titration charactersitics of the reconstituted membrane are almost indistinguishable from the unbleached membrane, and it has also a similar pK, 9.5–10, for the depletion with the maximum at 550–560 nm. The reconstitution of the bleached membrane was only 70% complete, and the

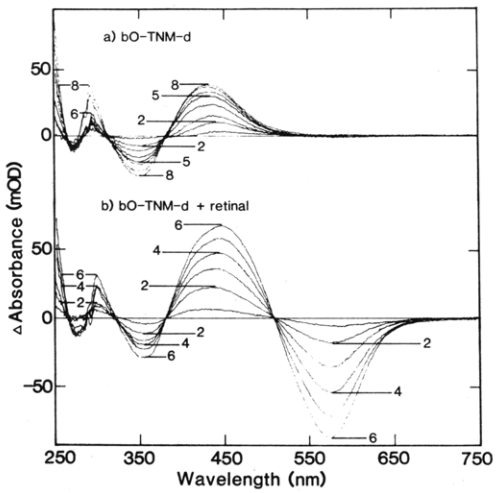

FIGURE 8: pH titration of (a) apomembrane from bR-TNM-d and (b) after subsequent reconstitution with *all-trans*-retinal and dark adaptation as in Figure 4. The difference to the pH 7 spectra was plotted for pH (1) 7.7, (2) 8.6, (3) 9.1, (4) 9.75, (5) 10.3, (6) 10.9, (7) 11.35, and (8) 11.7 in (a) and the difference to pH 6.1 spectra for pH (1) 7.3, (2) 9.0, (3) 9.85, (4) 10.6, (5) 11.2, and (6) 11.6 in (b).

remaining 360-nm depletion is accounted for by the nonregenerated membrane.

The apomembranes of bR and bR-TNM-d were similarly titrated before and after addition of *all-trans*-retinal. The apomembrane of bR did not show any spectral changes upon alkalinization at wavelengths above 330 nm (data no shown). The absorbance changes for the bleached bR-TNM-d (Figure 8a) were similar to those expected from nitrotyrosine with a pK of 9.5, which is 1 unit lower than in the unbleached membrane. After reconstitution with retinal, the titration spectra (Figure 8b) are indistinguishable from those prior to bleaching (Figure 5a). The pK for the nitrotyrosine (360-nm depletion) was shifted back to about 10.4 and was the same as for the alkaline chromophore transition in this sample. Note that there was no detectable tryptophan perturbation in the bleached sample. The spectral changes in the different membrane preparations were up to pH ~12 reversible and not time dependent. The spectra at pH ≥12 were taken immediately after alkali addition.

Discussion

Tetranitromethane has been shown to react rapidly with tyrosine at high pH but only slowly below pH 7 (Sokolovsky et al., 1966). The pH dependence suggests that the phenolate anion is the reactive species in the nitration reaction and the reaction is believed to proceed via a charge transfer complex between the phenoxide ion with TNM, followed by electron transfer from the phenoxide ion to TNM and scission of the N–C bond of TNM as the rate-determining step (Bruice et al., 1968).The coupling of the two radicals (NO_2· + xPO·) is expected to be very fast and not rate controlling. Indeed, we did not observe any reaction of TNM with bR at pH <7 in the dark. Absorption changes of pm at alkaline pH indicate a pK for tyrosines around pH 12 and possibly one tyrosine with a lower pK, between pH 10 and 11 (Bogomolni et al., 1978; Scherrer & Bogomolni, 1982). Reaction of pm with TNM at pH 7–11 and molar ratios of bR:TNM = 1:10 or less showed that at pH 9 tyrosine-26 was preferentially nitrated. The reason may be a lower pK of its phenolic hydroxyl group or higher accessibility to TNM than in the other tyrosines or both. Tyr-26 appears to be exposed to the aqueous environment since its nitro derivative shows spectral changes upon pH titration similar to those of nitrotyrosine in aqueous medium (Sokolovsky et al., 1966). Furthermore, nitrotyrosine-26 could be reduced to aminotyrosine with sodium dithionite, a lipid-insoluble ionic reducing agent. The pK values found for tyrosine and its nitro derivatives are higher in the membrane than in free amino acids and are most likely the results of their microenvironment (e.g., electronegative field).

Modification of tryptophan with TNM alters the spectral properties of the tryptophanyl residue markedly (Cuatrecasas et al., 1968) and was mainly seen when high ratios of TNM were used (Muhlrad et al., 1968; Katsura et al., 1982). We did not detect any such absorbance changes in the UV absorption during the modification or in the isolated peptides. Furthermore, it was possible to reduce the molar ratios of bR:TNM to 1:3, which resulted in a somewhat slower reaction rate but no significant difference in the membrane characteristics or the peptide map were detected. In addition, the amino acid analysis did not show a significant change in the tryptophan content after modification.

The absorbance changes during pH titration of pm containing nitrotyrosine or aminotyrosine in position 26 provide information about the chromophore structure. Nitration of this tyrosine lowered the pK of the alkaline transition, and subsequent reduction of the nitro- to an amino group increased it to the values expected for nitro- and aminotyrosines in an aqueous environment. We conclude that the alkaline transition of the chromophore correlates with and probably depends on deprotonation of tyrosine-26.

Indications for tyrosine deprotonation during the photocycle have been reported by several groups (Hess & Kuschmitz, 1979; Bogomolni et al., 1978; Scherrer & Bogomolni, 1982; Kalisky & Ottolenghi, 1982). We found that illumination of pm exposed to TNM at pH 6.0 shifted the absorption maximum of the purple complex to 535 nm and selectively nitrated tyrosine-64. Titration of this preparation revealed a decrease in the alkaline transition pK by about 2 units. However, even though nitrotyrosine-64 has a pK similar to that of the alkaline chromophore transition, the protonation state of this tyrosine does not seem to change or affect the transition. Reduction to aminotyrosine, which increases the phenolic hydroxyl pK to >11, does not alter the chromophore transition pK. Nitration of tyrosine-64 must cause other changes in the chromophore environment independent of the Tyr pK. Another indication of these changes is the blue shift of the bR chromophore below the alkaline transition. Nitrotyrosine-64 shows an absorbance at 360 nm only in the apomembrane but not when retinal is present. The absorbance at 430 nm of its ionized form does not change in the presence of the retinal. Nitrotyrosine-64 seems to be in an aqueous environment, because it can be reduced with sodium dithionite; nevertheless, the greatly diminished 360-nm absorbance suggests a direct interaction of tyrosine-64 with the retinal. However, interaction with a protein residue cannot be ruled out, because such an interaction could be disrupted by a structural change in the apomembrane when the chromophore is removed.

The cause of the chromophore blue shift by this light-induced nitration remains unexplained. A similar shift was seen after iodination of bR (Scherrer et al., 1981). Such a blue shift can be caused by generating a negative charge near the Schiff base, by removing a positive charge, and/or by deprotonation of the Schiff base nitrogen (Blatz et al., 1972). The pK for nitrotyrosine-64 of about 9.6 is too high to provide a negative charge at neutral pH, and also the reduction to aminotyrosine which increases this pK even more (pK > 11) did not change the chromophore absorption. Furthermore,

acidification of the membrane suspension to pH 2.5 did not result in an absorption change at 360 nm and shifted the absorption maximum of the chromophore to 565 nm, similar to the red shift to 605 nm of unmodified bR at about the same pH (data not shown). Therefore, the blue-shifted chromophore is not caused by the phenolate ion. The cause for the acid-induced red shift is probably the protonation of a carboxyl group near the Schiff base nitrogen (Mowery et al., 1979). Since the extent of the acid-induced red shift in nitrated bR is about the same, this carboxyl group is apparently not affected by the tyrosine nitration. If the blue-shifted chromophore were caused by a deprotonated Schiff base nitrogen, one would expect that upon acidification it protonates and a large red shift would be observed. Monomerization, which may cause a small blue shift (Dencher & Heyn, 1978; Casadio et al., 1980; Casadio & Stoeckenius, 1980), can be excluded because the nitrated membrane still shows the crystalline structure (K. Downing, personal communication). We can exclude an effect of TNM on the retinal, because the bleached and reconstituted membrane shows a similarly blue-shifted chromophore and the retinal chromophore extracted from bR-TNM-1 has an absorption spectrum similar to that extracted from native bR. At present, no definite interpretation for the chromophore perturbation can be given. We can only say that the nitration of tyrosine-64 causes a structural change affecting the chromophore environment which cannot be reversed by reducing the nitro group to the amino group.

Lemke & Oesterhelt (1981a) recently reported that the nitration of tyrosines-26, -64, -131, and -133 with a 100-fold excess of TNM at pH 8.0 caused a blue shift of the chromophore to 532 nm, an alkaline transition with a pK of 9, and a loss of lattice structure in the nitrated pm. After reduction with dithionite, only tyrosine-26 remained unreduced, and the dithionite reduction did not reverse any of these effects. They concluded that nitration of tyrosine-26 causes these changes. The effects they observed, except the loss in crystallinity, are essentially the same we see when only tyrosine-64 is nitrated, but they are clearly different from the ones we see when tyrosine-26 is nitrated. However, their observations are entirely compatible with our conclusion that tyrosine-64 causes these changes because we have shown that they cannot be reversed by reduction.

The high specificity of our nitration allowed us to clarify the role of tyrosines-26 and -64. Because both preparations retain their crystalline structure, valuable information might be gained by studying the three-dimensional structure of these membranes. The aromatic amino group allows further useful modifications. Studies on functional aspects with these specifically modified bR molecules are currently under way.

Acknowledgments

We are grateful to Dr. Stanley C. Rall, Jr., and Reed Harris at Gladstone Foundation Laboratories for carrying out the sequence determination of the CNBr fragments.

Registry No. N-Tyr, 621-44-3; Tyr, 60-18-4.

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Membrane Binding Properties of Blood Coagulation Factor V and Derived Peptides†

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ABSTRACT: The interactions of factor V and factor Va light chain with phospholipid vesicles were compared. The results showed that the factor Va light chain bound with the same parameters as factor V when the proteins were present at similar densities on the membrane. The protein–vesicle collisional efficiency was 30–50% for both factor V and factor Va light chain. The factor Va light chain bound at a higher density, and the additional binding interactions had lower affinity. The dissociation process showed negative cooperativity, possibly due to competition for acidic phospholipids in the membrane. The higher molar packing density produced more rapid protein–membrane dissociation rate constants. However, when factor V and Va light chains were present at similar molar densities on the vesicle, the dissociation rates, estimated by two methods, were similar. Analysis of dissociation rates also showed that factor Va interacted with factor Xa on the membrane surface while factor Va light chain did not. Factor Va generated by thrombin digestion of factor V did not result in a major loss of membrane-bound protein mass unless ethylenenediaminetetraacetic acid was present; in the latter case the mass changes indicated that all peptides were removed from the membrane except factor Va light chain. Equilibrium and dynamic measurements showed that ionic strength had a major effect on the dissociation rate but not on the association process. The salt effect indicated interaction between oppositely charged species with the product of the number of charges equal to at least −5.5. Factor Va light chain appeared to interact with phospholipids via a general charge interaction rather than via a specific charge stoichiometry. Lysine modifications readily prevented protein–membrane binding while tryptophan modification had little effect.

Blood coagulation factor Va interacts with factor Xa on membrane surfaces to form the prothrombinase complex [see Jackson & Nemerson (1980) for a review]. All three proteins involved in this reaction bind to membranes containing acidic phospholipids (Papahadjopoulos & Hanahan, 1964; Nelsestuen et al., 1976; Bloom et al., 1979). Thorough understanding of the mechanism of the prothrombinase reaction requires knowledge of the physical interactions between each of the components and how these participate in ternary and quaternary (factor Va, factor Xa, prothrombin, and phospholipid) macromolecular assemblies. The three proteins each exist as inactive precursors, as active, proteolytically cleaved proteins, and as isolated protein fragments with partial functions. One important question is whether the protein–membrane interactions are affected by these cleavages and whether the changes affect the prothrombinase reaction. Factors X and Xa appear to have identical membrane binding properties (Nelsestuen et al., 1976; Van de Waart et al., 1983). Prothrombin and its isolated 156-residue amino-terminal fragment display at most small differences in their membrane interactions (Nelsestuen, 1976).

Factor V has been studied recently and the conclusions are inconsistent. Bloom et al. (1979) reported a K_D for the factor V–membrane complex of about 10^{-7} M, and Higgins & Mann (1983) reported that factor Va light chain bound somewhat less tightly. Pusey et al. (1982) reported that assumptions made in these studies were not valid under similar circumstances and that the dynamics of factor V–membrane binding indicated a K_D of about 10^{-10} M. First-order rate plots for dissociation of the complex were not linear so this was an average value (Pusey et al., 1982). This tighter binding correlated closely with reported binding of factor Va to cell membranes (Tracy et al., 1981; Kane & Majerus, 1982; Tracy et al., 1983). The discrepancy in the K_D for factor V–phospholipid vesicle binding is therefore important for interpretation of factor V–cellular interactions; the tighter binding constant implies that factor V–cellular interactions may be mediated by phospholipids alone while the weaker binding constant strongly implies that other forces (e.g., a cell surface protein receptor) are involved in factor V–cellular interactions.

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0006-2960/84/0423-6202$01.50/0