# REACTIVITY OF TRYPTOPHANS IN RHODOPSIN

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SUMMARY: Oxidation with N-bromosuccinimide detects a total of about ten tryptophan residues in detergent-solubilized bovine rhodopsin. One of these tryptophans is more reactive in bleached than in unbleached rhodopsin, suggesting its involvement in the chromophore binding site. Oxidation of this residue is accompanied by loss of the 500nm. absorbance in unbleached rhodopsin. Similar experiments with bacteriorhodopsin are inconclusive.

The photopigments of vertebrate photoreceptors and of certain halophilic bacteria display some remarkable similarities (1), and several recent reports have indicated the involvement of aromatic amino acid residues in the chromophore binding sites of both rhodopsin and bacteriorhodopsin (2,3,4). We present here chemical evidence for the interaction of a tryptophan residue with the retinal moiety of detergent-solubilized bovine rhodopsin, based on the reactivity to N-bromosuccinimide (NBS)[1] of bleached and unbleached samples.

MATERIALS AND METHODS: Bovine rod outer segments, prepared from frozen cattle retinas (Hormel, U.S.A.) by the method of Papermaster & Dreyer (5), were used immediately or stored frozen in 27% sucrose, 10mM Tris acetate pH 7.4, until use. SDS-acrylamide gel electrophoresis of samples, both before and after NBS treatment, showed only a major band corresponding to rhodopsin. Outer segment membranes were solubilized in 1% (v/v) Emulphogene BC 720 (General Aniline Film Corp.), 0.1M Tris acetate, pH 7.4 ( final concentrations), to give absorbances in the range 0.2-0.3 at 500 nm. Bleached solutions were obtained by exposure to room light for a few minutes, after which the 500nm. absorbance was replaced completely by a peak at 365nm., corresponding to the N-retinylidene opsin (indicator yellow) photoproduct (8). Rhodopsin concentrations were determined from the 500nm. absorbance of unbleached samples, using $\epsilon_{500} = 41,000$ (10).

Bacteriorhodopsin was prepared by the method of Oesterhelt and Stoeckenius (6) from a vacuole-free strain of Halobacterium halobium (National Research Council, Canada, strain #34003).

N-bromosuccinimide (Fisher) was recrystallised from water before use. All-trans retinal (Sigma) and N-acetyl-L-tryptophan ethyl ester (Mann)

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[1]Abbreviations: NBS, N-bromosuccinimide; SDS, sodium dodecylsulphate.



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were used without further purification. Unless otherwise specified, all manipulations of photosensitive material were performed in the dark or dim red light.

Oxidation of tryptophan residues was followed in the Cary model 14 spectrophotometer by addition of small aliquots (usually 10 µl) of NBS, dissolved in the appropriate buffer, to both sample and reference cuvettes at 25 C. Sample and reference compartments of the spectrophotometer were thermostatted and flushed with dry $N_2$. At least 20 minutes were allowed for completion of reaction after each addition of NBS, during which time the slits of the spectrophotometer were closed to prevent irradiation of the sample. The extent of Trp oxidation at each stage was calculated from the decrease in absorbance at 280nm., after correction for dilution, by the method of Spande and Witkop (7). Control experiments included the reaction of NBS with free tryptophan (as the N-acetyl ethyl ester) and with free retinal, in 1% Emulphogene, pH 7.4 .

RESULTS AND DISCUSSION: Reaction of NBS with free tryptophan in neutral detergent buffer is almost stoichiometric, and is indistinguishable from the same reaction in the absence of detergent and at lower pH. Free retinal reacts with NBS at low concentrations with a marked decrease in the 380nm. absorbance peak and a gradual shift to lower wavelengths. The observations

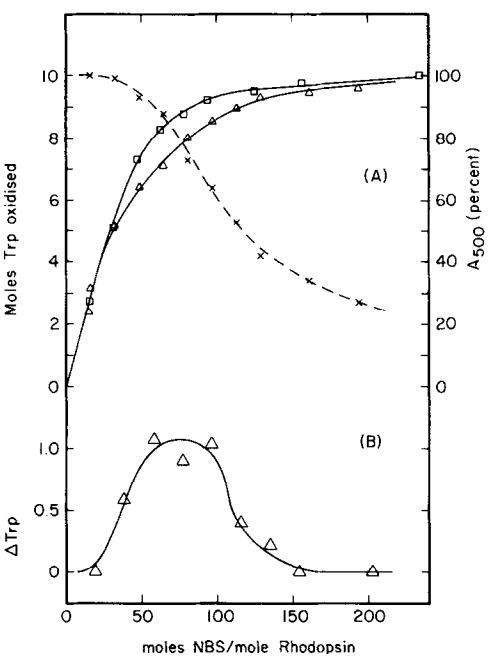


FIGURE 1: (A) NBS oxidation of tryptophan in bleached(□) and unbleached(Δ) rhodopsin, and variation of 500nm. absorbance(x). 1% Emulphogene, 0.1M Tris acetate, pH 7.4, 25°C.
(B) Difference titration with NBS of bleached and unbleached rhodopsin. ΔTrp = bleached - unbleached .

are consistent with oxidation of the conjugated polyene to form a complex mixture of products. No change in absorbance at 280nm. was observed on bleaching of rhodopsin solubilized in Emulphogene.

The course of oxidation of tryptophans in rhodopsin with increasing amounts of NBS is shown in Figure 1A. Oxidation proceeds with a decrease in absorbance at 280nm. and a simultaneous increase at 250nm., and shows an isosbestic point in the region of 265nm. as expected for simple oxindole formation (7). A maximum of about 10 ($\pm$1) tryptophans per mole of rhodopsin are detected for both bleached and unbleached samples. Heller (9), using NBS titration, reports a value of about 5 for the Trp content of cattle rhodopsin, but this is based on an $\epsilon_{500}$ of 23,000 which seems to be in error (11). Correction to the accepted $\epsilon_{500}$ of about 41,000 (10) gives 9 Trp per rhodopsin. Estimation from the $\epsilon_{280}$ of purified rhodopsin (11), after correction for tyrosine absorbance, gives a value of about 10 for the Trp content.

Comparison of the course of oxidation of bleached and unbleached samples (Fig.1A) shows a marked, and reproducible, difference in the reactivity to NBS in the region of 50-100 moles NBS/mole rhodopsin. This difference amounts to about one tryptophan per rhodopsin molecule, and is illustrated more clearly by the difference titration shown in Figure 1B. In this experiment bleached and unbleached samples of rhodopsin, placed in the reference and sample compartments of the spectrophotometer respectively, were titrated simultaneously with NBS so that the difference in extent of tryptophan oxidation could be monitored directly. Once again, the unbleached sample shows a single less reactive tryptophan residue in the region of 50-100 moles NBS/mole rhodopsin.

Concurrently with oxidation of tryptophan in unbleached rhodopsin there is a decrease in the absorbance at 500nm. (Fig.1A, dashed line). The 500nm. band is progressively replaced by a peak of similar height at about 360nm., and spectra show an isosbestic point in the region of 415nm. NBS treatment has little, or no, effect on the 365nm. chromophore absorbance of bleached rhodopsin. It thus appears that, unlike free retinal under the same conditions, the chromophore in both bleached and unbleached rhodopsin does not react directly with NBS, at least upto about 200 molar excess. The maximal decrease in 500nm. absorbance of unbleached rhodopsin correlates reasonably well with oxidation of the less reactive tryptophan.

Thus, there appears to be a single tryptophan residue in rhodopsin which is protected from NBS oxidation in the unbleached state. Oxidation of this residue at higher NBS concentrations results in chemical bleaching of the pigment. The most straightforward interpretation of these observations

is that this tryptophan forms part of the retinal binding site of rhodopsin, and that isomerization and/or displacement of the chromophore by light results in increased exposure and, hence, increased reactivity of this Trp. We cannot, of course, rule out the possibility of more subtle effects related to conformational changes in rhodopsin induced by bleaching, which might result in altered reactivity of Trp residues remote from the chromophore site. However, the simplest interpretation is in accord with electron spin resonance (3) and Raman spectroscopy (2) observations which indicate the direct involvement of aromatic residues with the rhodopsin chromophore.

N-bromosuccinimide is usually most reactive to the indole moiety of tryptophan residues, though various other amino acids may also react without interfering with the tryptophan determination (7). Complete oxidation of Trp in rhodopsin requires an excess of NBS (~15 moles/mole Trp) which is somewhat higher than that normally required for soluble globular proteins (7), and it is possible that other residues in rhodopsin are reacting and competing for the NBS. Alternatively, the tryptophans of this membrane associated protein may well be intrinsically less accessible than the same groups in soluble proteins. It is also possible that NBS is reacting with the lipid fraction of the solubilized rod outer segment membranes, though we see no large increases in U.V. absorbance which might be expected for lipid oxidation.

Bacteriorhodopsin shows many remarkable similarities with vertebrate rhodopsins (1), though the molecules are of very different biological origin and show no immunological cross-reactivity (C.A. Converse, personal communication). Mendelsohn (4) has proposed, on the basis of resonance enhanced Raman spectra, that the chromophore of bacteriorhodopsin involves a charge transfer complex between retinal and a nearby tryptophan group on the protein. Unfortunately, experiments on the reaction of NBS with bacteriorhodopsin have proved inconclusive. Although treatment of both light- and dark-adapted samples with NBS, under a variety of conditions, results in a decrease in the 560-570nm. absorbance of the bacteriorhodopsin, this occurs by a gradual shift of the chromophore absorbance to lower wavelengths, and eventually into the U.V., suggesting direct oxidation of retinal. Furthermore, the behaviour of the absorbance at 280nm. is complex, and seems to involve various competing effects in addition to oxindole formation, such as oxidation of lipids or retinal, together with changes in light scattering. Under these conditions it is not possible to come to any conclusions about the tryptophan residues in this molecule.

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