Aqueous Cyanohydridoborate Reduction of the Rhodopsin Chromophore*

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The imino linkage between 11-cis retinal and the lipoprotein opsin has been reduced under mild conditions (aqueous, 3°C., pH = 5) by the reagent cyanohydridoborate. The sole retinyl derivative, recovered in 20% yield after basic hydrolysis, was identified as N-retinyl lysine.

The major chromophore of both vertebrate and invertebrate lipo-protein visual pigments is derived from 11-cis retinal (vitamin A aldehyde) (Oroshnik, et al., 1956; Hubbard, et al., 1958; Hara, et al., 1967). The absorption of light by this retinylidene chromophore and its isomerization is the primary act of the visual process.

It was shown that in bovine rhodopsin that, after exposure to light, the retinylidene chromophore was linked as a Schiff base to a lysine side chain which could be reduced using sodium borohydride (Bownds, 1967; Akhtar, et al., 1968). However, rhodopsin which has not been light-exposed can be reduced by borohydride in aqueous media only at temperatures above 60°C, where we have found the chromophore bound only as N-retinyl phosphatidyl ethanolamine (Poincelot et al., 1969, 1970). Similarly, the acidified methanol extraction of lyophilized bovine rods or detergent micelles of rhodopsin brings the chromophore into solution essentially quantitatively as a protonated form of the Schiff base, N-retinylidene phosphatidyl ethanolamine (Poincelot, et al., 1969, 1970). When the same experiment was performed after exposure to light the bulk of the chromophore was found on lysine, consistent with the earlier

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results suggesting its transiminization from lipid to protein. The idea of a lipid for the chromophore binding site, however, has been challenged (Hall, et al., 1970; Anderson, 1970; Borggreven, et al., 1970) and similar experiments performed in our own laboratory on squid rhodopsin showed a protein binding site, both before and after light exposure (Fager, et al., Fed. Proc., 1971).

In light of this contradictory evidence, we have reinvestigated the question of the native binding site of vertebrate rhodopsin using cyanohydridoborate, ($NaBH_3CN$, also known as sodium cyanoborohydride), a borohydride derivative more hydrophobic, more specific for protonated Schiff bases, and more stable at acid pH's than is sodium borohydride itself (Jencks, personal communication; Borch, et al., 1971).

Sodium cyanohydridoborate was obtained from ALFA INORGANICS and was purified by the method of Borch (Borch, et al., 1971). 10^{-4} M Bovine rhodopsin solubilized in 1% emulphogene and buffered with 1 M sodium acetate, pH 5.0 was incubated with 50 mg/ml sodium cynoborohydride and stirred in the dark at 3°C. After 48 hrs greater than 95% of the absorbance at 500 nm had disappeared and was replaced by an absorbance maximum at 325 nm, indicating the chromophore had been reduced. This reduction is associated with the more effective reducing action of cyanoborohydride at the Schiff base linkage under acid conditions and not to a non-specific denaturation of the rhodopsin since rhodopsin kept under the same buffer conditions without cyanoborohydride and rhodopsin incubated with the same cyanoborohydride concentration but buffered at pH 7.0 both showed negligible disappearance of the 500 nm band.

The reduced rhodopsin was lyophylized, extracted 2 times with hexane to remove emulphogene and hydrolyzed with 2N NaOH for 5 hours at 110°C in sealed evacuated combustion tubes. The hydrolysate was washed from the tubes with an additional volume of distilled water; chloroform and methanol were added to bring the proportions to 2:1:1, C:M:W by volume. This mixture was shaken and the phases allowed to separate. The lower (chloroform) phase contained the

retinyl derivative. This phase was clarified with $MgSO_4$, and a spectrum was taken. In two experiments yields of 20 and 21% were obtained based on ε_{500} of 42,000 for rhodopsin and ε_{325} of 50,000 for the reduced Schiff base. The chloroform phase was concentrated on a rotary evaporator for thin layer chromatography.

Thin layer chromatography on Brinkmann silica G analytical chromatography plates showed a single major spot which was (1) fluorescent, (2) showed a positive Carr-Price reaction characteristic for retinyl derivatives, (3) a ninhydrin test characteristic for primary amines, and (4) a match of the R_f of N retinyl lysine in four solvent systems, (chloroform:methanol:30% ammonium hydroxide, 70:30:4, methylethyl ketone:pyridine:acetic acid: water, 70:15:15:2, butanol:acetic acid:water, 4:1:1, chloroform:methanol, 10:1). A faint additional spot was carried by the residue of emulphogene in the sample in some experiments. There were no spots matching N-retinyl derivatives of ethanolamine or serine which would have been obtained had the retinyl group been bound to an amino phospholipid.

These experiments support the contention that the retinylidene group is bound to lysine in native bovine rhodopsin and contrasts with earlier experiments from this laboratory involving acid methanol extraction and aqueous borohydride reduction above 60°C. Other laboratories after borohydride reduction have found the chromophore bound to lysine amino groups under some conditions and to lipid under others (Hirtenstein and Akhtar, 1969; Zorn, 1971; Girsc et al., 1971). In aqueous reductions with cyanohydridoborate we find only lysine bound chromophore.

It is apparent from all the attention given this problem by our laboratory and others that the chemistry of the chromophore linkage in native rhodopsin is quite subtle and despite the seemingly convincing weight of evidence now favoring a lysine-protein binding site for the chromophore an unequivocal answer to this question based on chemical evidence alone seems highly unlikely.

It is our opinion that a final answer requires an observation of the linkage *in situ* by appropriate physical methods.

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