A Water-soluble Polylysine-Retinaldehyde Schiff Base
STABILITY IN AQUEOUS AND NONAQUEOUS ENVIRONMENTS*

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In order to improve the existing models of retinal-protein Schiff bases, a water-soluble polylysine-retinaldehyde imine has been synthesized and its stability assessed under a variety of conditions through changes in the visible absorption spectrum. The compound absorbs at 342 nm and consists of a 90-kDa poly-L-lysine containing a retinal Schiff base in about 2% of the lysyl ε-amino ends. Retinal is mostly in the all-trans form; under no conditions is more than 15% of the 13-cis isomer detected. The absorption maximum exhibits a pH-dependent reversible shift to 402 nm, with an apparent pKₐ ≈ 3.4. In the presence of the anionic surfactant sodium dodecyl sulfate, this pKₐ is shifted to ≈8.9, probably because of electric neutralization of lysyl ε-amino groups. Other detergents (cetyltrimethylammonium bromide, Triton X-100) do not modify the Schiff base pKₐ, but rather promote its hydrolysis; in this case detergents act in the same way as certain solvent mixtures, by providing an amphiphilic environment to the imine that in turn stabilizes the products of hydrolysis. Our results suggest that once the surfactant reaches the Schiff base, preferential partition of retinal into detergent micelles is the main factor facilitating imine bond breakdown. The response of our synthetic Schiff base to changes in pH or solvent polarity point together to an important role of the supporting polypeptide in providing a suitable environment to the chromophore.

Retinaldehyde is often found in nature as the prosthetic group of proteins (rhodopsins) to which it is linked via a Schiff base, usually to the ε-amino group of a Lys residue (Lanyi, 1984). Of these proteins, bacteriorhodopsin from Halobacterium purple membranes has received particular attention, in view of the simplicity of its purification and its potential applications (Oesterhelt and Stoeckenius, 1971; Skulachev, 1979). The possibility of using this light-driven protein pump in semisynthetic systems has focused the attention of many scientists on the stability and other properties of the Schiff base through which retinaldehyde is bound to the apoprotein. Studies have been carried out using (a) the native system, e.g. the work by Caplan and co-workers (Eisenbach and Caplan, 1979; Eisenbach et al., 1979) on the stability of bacteriorhodopsin in organic solvents; (b) reconstituted bacteriorhodopsins, in which retinaldehyde has been substituted by other aldehydes following different procedures (Schreckenbach et al., 1977; Bayley et al., 1981; Crouch, 1986; Spudich et al., 1986; Fendler et al., 1987; Gänser et al., 1988; Koutalos et al., 1989); and (c) fully synthetic systems, formed by retinaldehyde or retinaldehyde analogues and various amines (Blatz et al., 1971; Waddell et al., 1973; Johnston and Zand, 1973; Das et al., 1979; Bassov and Sheves, 1986; Sheves et al., 1986).

In the lines of the latter studies, the present work deals with a synthetic retinaldehyde-poly-L-lysine Schiff base. By a slight modification of the method of Johnston and Zand (1973), we have synthesized a fully water-soluble compound and studied its stability under a variety of conditions, including pH, organic solvents, and surfactant solutions.

MATERIALS AND METHODS

Poly-L-lysine hydrobromide (≈430 Lys residues/molecule, M₉ ≈ 90,000), all-trans-retinal, and the various surfactants were purchased from Sigma. Double-distilled water was used throughout this study. All organic solvents were thoroughly dried and freshly redistilled before use.

All procedures were carried out in the dark or under dim light. Poly L-lysine hydrochloride was synthesized from the hydrobromide by extensive dialysis against 0.1 M HCl; this step also allows elimination of low molecular weight polyanionic acid chains. The Schiff base was synthesized as follows: poly-L-lysine hydrochloride and all-trans-retinal, at a 54:1 Lys:retinaldehyde mole ratio, unless otherwise indicated, were allowed to react as indicated by Johnston and Zand, (1973). Once the reaction had taken place, the mixture was brought to pH 8 and freeze-dried; the resulting powder was easily dissolved in water at room temperature. It was found that adjusting the pH to 8 was essential for obtaining a water-soluble product after freeze-drying.

Absorption spectra were recorded in a Uvikon 860 Kontron spectrophotometer using 1-ml quartz cuvettes. The Schiff base concentration was 80 μM unless otherwise specified. The slit width was 2 nm; the scan speed varied between 100 and 500 nm/min; 1-nm recording intervals were used; lamp change occurred at 300 nm. Data were processed with a built-in CPU 8088 Kontron computer. pH measurements were carried out in a MicropH 2002 Crison pH meter, using a 9811 Ingold combination electrode. Unless otherwise specified, pH was adjusted by direct addition of 12 N NaOH or HCl from micro-syringes. Retinal isomers were analyzed essentially as described by Scherrer et al. (1989).

RESULTS

Stability in Pure Water—The water-soluble Schiff base displays an absorption maximum at 342 nm. Changing the Lys:retinaldehyde mole ratio from 54:1 to 540:1 did not cause variation of the absorption maximum or of the apparent molar absorption coefficient, beyond the limits of experimental error, suggesting that even at 54:1, retinyl moieties are not clustered forming separate domains. When the Schiff base is reduced with NaBH₄, a marked decrease in absorbance is observed, as expected from the chemical modification of the chromophore. Bleaching after addition of the reducing agent is a slow process, taking place with a t½ of the order of 1 h;
the surface of poly-L-lysine, but also within the polypeptide core. Changes in absorbance at 342 nm can be used to monitor the stability of the compound as a function of time. It appears required to produce a 10% decrease in absorbance.

Retinal Isomerization—The extent of retinal photoisomerization was examined under the various experimental conditions, by organic solvent extraction and HPLC, as described under “Materials and Methods.” All-trans-retinal, as supplied by the manufacturers, is at least 99% pure. However, the freshly synthesized polylysine-retinal Schiff base contains 11.5 ± 1.3 13-cis form (average ± S.D., n = 4). This proportion does not vary significantly after the different measurements. For instance, after recording 10 successive spectra in the 240–520 nm range, the percentage of 13-cis-retinal is 12.0 ± 0.5; after lowering the pH to 2.6 and recording one spectrum, the 13-cis proportion is 15.0 ± 4.7.

pH Effects—In these experiments aliquots of a Schiff base solution were mixed with equal volumes of the appropriate solutions of NaOH or HCl and spectra recorded immediately afterwards. Then the exact pH of each sample was measured. Results are shown in Fig. 1; a new species (λmax = 402 nm) is observed at low pH that disappears after realkalinization of the samples. The presence of an isosbestic point suggests that only two chemical species are implicated in the process, whose apparent pKs occurs at 3.4 (Fig. 1B). The species absorbing at 402 nm is putatively identified as the protonated Schiff base.

When the spectra are not recorded immediately after pH adjustment, an isosbestic point is no longer observed, and the sample absorbances decrease in a process that reaches equilibrium in a few hours. The percent decrease in absorbance (measured at the respective λmax) was studied as a function of pH, 4 h after pH adjustment; maximum loss is observed in the pH range 3.8–4.0, near the apparent pK of the Schiff base (data not shown).

Effect of Nonaqueous Solvents—For these experiments, the freeze-dried Schiff base was dissolved in the appropriate solvent, or solvent mixture, to a final concentration of 80 μM and left in the dark for 24 h, with stirring. The samples were then centrifuged to remove any insoluble material and their absorption spectra recorded immediately afterwards. Samples of pure all-trans-retinal were subjected to precisely the same treatment for each solvent. Solvents were mainly selected among those used by Caplan and co-workers (Eisenbach and Caplan, 1979; Eisenbach et al., 1979) in their studies with native purple membranes, including examples in which the Schiff base was or was not easily hydrolyzed. The absorption maxima of the Schiff base and retinaldehyde solutions are collected in Table I. Following the criteria of the authors mentioned above, we suggest that those solvents in which both the Schiff base and retinal display the same absorption maximum, e.g. chloroform, allow breakdown of the imine bond. (In this particular case, a white-yellowish precipitate was formed, consisting mainly of poly L-lysine). The imine bond would, on the contrary, remain partially or totally intact with those solvents, e.g. methanol, in which the absorption maximum of the Schiff base is clearly different, usually blue-shifted, with respect to all-trans-retinal. Methanol solutions gave initially nonreproducible results; it was found that they were particularly sensitive to light and humidity. The behavior of methanol-water mixtures was very interesting, and our results are shown in Fig. 2. A 9/1 (v/v) methanol:water mixture produces complete hydrolysis of the Schiff base, which is partially or totally preserved with most other solvent compositions. 10 μl of 12 M HCl were added to the cuvettes containing the dissolved Schiff base, to a final pH ≈ 1.5; the pH behavior, consisting of a red shift at acidic pH, was similar to that seen for the Schiff base in aqueous solution (Fig. 1) and occurred at all methanol:water mixtures, except at 9:1, (Fig. 2), thus confirming that the latter is particularly active at promoting hydrolysis of the imine bond.

Stability in Surfactant Solutions—The study of the stability of Schiff bases in the presence of surfactants is very pertinent since the latter are widely used in the isolation and purification of lipid-bound rhodopsins. Triton X-100 was chosen as a representative nonionic detergent; it has, in addition, been often in use for bacteriorhodopsin solubilization (Reynolds and Stoeckenius, 1977; Doncher and Heyn, 1978). Aqueous solutions of the Schiff base were treated with various concen-

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1 The abbreviations used are: HPLC, high performance liquid chromatography, CTAB, cetyltrimethylammonium bromide, SDS, sodium dodecyl sulfate.
of $k_{app}$ = $7.9 \pm 0.13 \times 10^{-3}$ min$^{-1}$ (mean ± S.D. of six experiments, with surfactant concentrations ranging from 0.0125 to 0.20 M), corresponding to a $t_{1/2}$ ≈ 88 min. The observed independence of $k_{app}$ with surfactant concentrations suggests that Triton X-100 is not implied in the rate-limiting step of the hypsochromic process. The nature of this Triton-induced process is believed to consist of the hydrolysis of the Schiff base; this is supported by the facts that (a) all-trans-retinal in a 0.05 M Triton X-100 solution has an absorption maximum at 382 nm, the same as the Schiff base under those conditions; (b) chloroform extracts of detergent-treated Schiff base show an absorption band at $\approx 397$ nm, the same as all-trans-retinal in chloroform (Table I), whose intensity increases with surfactant concentration; and (c) the 382-nm species does not longer experience pH-dependent shifts.

Detergents other than Triton X-100, namely cetyltrimethylammonium bromide (CTAB) and sodium dodecyl sulfate (SDS), were used as representatives of the cationic and anionic surfactants respectively. At neutral pH, the behavior of CTAB is similar to that of Triton X-100; a new species, with a maximum absorption wavelength at 387 nm, is formed in the presence of detergent (data not shown). The anionic surfactant SDS, at concentrations up to $4 \times 10^{-3}$ M, produces precipitation of the Schiff base (Fig. 5). Surfactant concentrations equal or above $4 \times 10^{-3}$ M resolubilize the precipitates and produce a red shift, the new species absorbing from 431 ([$SDS$] = $4 \times 10^{-3}$ M) to 389 nm ([$SDS$] = 0.25 M). SDS also differs from the other two detergents in that it preserves intact the Schiff base. This is shown very clearly by the pH behavior of the imine in the presence of SDS (Fig. 6). The nonprotonated and protonated species appear now at 361–367 nm and 406–431 nm, respectively, depending on detergent concentration. Moreover, the apparent $pK_c$ is also changed by various units, to an average value of $8.9 \pm 0.50$ (mean ± S.D. of 14 measurements, at SDS concentrations ranging from $4 \times 10^{-3}$ M to $5 \times 10^{-3}$ M).

**DISCUSSION**

**pH Effects**—Our Schiff base shows an important pH-dependent reversible shift in its absorption spectrum going, on acidification, from 342 to 402 nm, with an apparent $pK_c$ of 3.4. Since no other group exists in our system that may be

**Fig. 3. Effect of Triton X-100 on the absorption spectrum of the polypeptide-retinaldehyde Schiff base.** Samples were incubated in the dark for 24 h in the presence of Triton X-100 at the molar concentrations indicated on each curve.

**Fig. 2. The maximum absorption wavelengths of the polylysine-retinaldehyde Schiff base and pure retinal in methanol-water mixtures.** ○, Schiff base incubated in the dark for 24 h with the corresponding methanol-water mixture; O, the same, but with addition of 10 μl of 12 N HCl/ml at the end of the incubation period; ▲, pure retinal.

**Table I.** Maximum wavelengths of the absorption spectra of (a) poly-L-lysine retinaldehyde Schiff base and (b) all-trans-retinal, in various solvents, and a comparison with their effects on freeze-dried purple membranes

<table>
<thead>
<tr>
<th>Solvent</th>
<th>λ_{max} (nm)</th>
<th>Effect on purple membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>342</td>
<td>None</td>
</tr>
<tr>
<td>Chloroform</td>
<td>391$^a$</td>
<td>Negligible</td>
</tr>
<tr>
<td>Benzene</td>
<td>380</td>
<td>Negligible</td>
</tr>
<tr>
<td>Toluene</td>
<td>374</td>
<td>Negligible</td>
</tr>
<tr>
<td>Methanol</td>
<td>360$^d$</td>
<td>Retinal release</td>
</tr>
<tr>
<td>2-Propanol</td>
<td>383</td>
<td>Retinal release</td>
</tr>
<tr>
<td>Diethyl ether</td>
<td>370$^d$</td>
<td>Negligible</td>
</tr>
<tr>
<td>Dioxane</td>
<td>369$^b$</td>
<td>Negligible</td>
</tr>
<tr>
<td>Acetone</td>
<td>376$^f$</td>
<td>Negligible</td>
</tr>
<tr>
<td>Heptylamine</td>
<td>362</td>
<td>Sparingly soluble</td>
</tr>
<tr>
<td>Triethylamine</td>
<td>363</td>
<td>Sparingly soluble</td>
</tr>
<tr>
<td>Chloroform-methanol, 1:1$^f$</td>
<td>350</td>
<td>None</td>
</tr>
</tbody>
</table>

$^a$ Data from Eisenbach and Caplan (1979).
$^b$ Insoluble.
$^c$ Abundant white-yellowish precipitate.
$^d$ Very sensitive to moisture and light. See text for details.
$^e$ Partial retinal release.
protonated in this pH region, we attribute the spectral change to Schiff base protonation. \( pK_a \) values around 6–7 have been determined for a 1-butylamine-retinal Schiff base (Blatz et al., 1971; Schaffer et al., 1975). In bacteriorhodopsin, a comparable phenomenon is observed with a \( pK_a \approx 13.3 \) (Druckmann et al., 1982). The high \( pK_a \) in purple membranes have been explained variously, invoking the relatively polar microenvironment that surrounds the protonated Schiff base in the protein (Warshel et al., 1984) or stabilization through hydrogen-bonding to water (Hildebrandt and Stockberger, 1984). \( pK_a \) values for synthetic retinal imines as low as 1.8 have been described (Sheves et al., 1986). The low \( pK_a \) found in our case may be due to the presence of a large number of positively charged lysyl \( \epsilon \)-amino groups in the neighborhood of the Schiff base; Baasov and Sheves (1986) have shown that the \( pK_a \) for retinal Schiff base protonation in bacteriorhodopsin is markedly reduced by the presence of a nonconjugated positive charge on the retinal in the vicinity of the Schiff base linkage. It is also known that the retinyl chromophore may be influenced by nearby ionizable groups (Muccio and Cassim, 1979). Moreover, our results (Fig. 6) show that in the presence of SDS, whose negative charges should counter the effect of Lys residues, the \( pK_a \) of our system is increased from 3.4 to 8.9. Druckmann et al. (1982) have suggested that a \( pK_a \) change of several units occurs in bacteriorhodopsin during the photocycle; this could be at least partially explained, according to our results, by a light-induced conformational change that would change the ionic environment of the chromophore.

**Solvent and Surfactant Effects**—The visible spectrum of rhodopsins is very sensitive to environmental changes. The effects of nonaqueous solvents (Eisenbach and Caplan; Eisenbach et al., 1979), surfactants (Casadio et al., 1980; McCaslin and Tanford, 1981; Padrós et al., 1984), and other amphiphiles (Nishimura et al., 1985) are well documented. Becker et al. (1976) studied the absorption spectra of a retinal-butylamine Schiff base in a variety of solvents; their imine appears to be particularly stable in all solvents used. We have examined the influence of nonaqueous solvents and surfactants on our synthetic Schiff base; the effect of various solvents is summarized and compared with the results using purple mem-

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**Fig. 4.** Kinetics of Triton X-100 effects on the absorption spectrum of the Schiff base. A, spectra recorded in the presence of 0.05 M surfactant, every 15 min for the first 9.5 h and then after 30 h of treatment. B, the maximum absorption wavelengths of the Schiff base, as a function of time. C, kinetics of loss of Schiff base, measured as a decrease in the Schiff base mole fraction \( x_{\text{bs}} \).
to some extent during incubation with the Schiff base, thus
to the fact that even freshly distilled solvents will be hydrated
in Table I. Accepting the interpretation of Eisenbach
et al. (1979) according to which the similarity of absorption
maxima between Schiff base and purple membrane are equivalent only with about
one-half of the solvents tried. This is probably an indication
that the protein microenvironment is at least as important as
the outer solvent for the stability of the imine bond, in
accordance with former predictions (Honig et al., 1976).

Surfactant action is probably exerted, as in the case of
solvents, through changes in the Schiff base environment,
rather than participating stoichiometrically in particular re-
actions. At least for Triton X-100, our kinetic studies suggest
this kind of involvement in the surfactant induced hydrolysis
of the Schiff base. Padrós et al. (1984) described that SDS
induces the appearance of the blue form of bacteriorhodopsin,
whereas Triton X-100 or CTAB do not so, suggesting, in
accordance with our results (Figs. 3 and 6), that SDS interacts
with the Schiff base in a different way than the other two.
There are data, however, indicating that the peculiarities of SDS
are mainly due to its effects on the apoprotein, rather
than on the Schiff base itself. Becker et al. (1986) indicate
that both Triton X-100 and SDS result in rapid hydrolysis of
a protonated 11-cis-retinal Schiff base with 1-butylamine. In
our system, we have already mentioned that the SDS-induced
pK\textsubscript{a} shift (Fig. 6) could be explained by SDS-lysyl electrostatic
interactions leading to a change in the imine ionomic
atmosphere. Moreover, McCaslin and Tanford (1981) have studied
the way in which detergents influence the recombination of
opsin with retinaldehyde. SDS, Triton X-100 or tetradecyl-
trimethylammonium bromide (very similar to our CTAB)
were unable to support rhodopsin recombination. This is the
opposite reaction to what we have considered, and, of course,
in their case SDS-retinal interaction is not hindered by the
protein, so that, as those authors conclude, the preferential
partition of retinal into detergent micelles may be the main
cause for the low recombination of rhodopsin solubilized in
certain surfactants.

Our observation that at least some detergents facilitate
imine hydrolysis provides a rationale for the gradual bleaching
of purple membrane in the presence of e.g. Triton X-100
(Casadio et al., 1980; González-Mañas et al., 1990). The mech-
nism of such process may be explained by the studies with
organic solvents. It has already been mentioned that as sug-
gested by Eisenbach and Caplan (1979), interaction with the
Schiff base requires both nonpolar and polar groups, in the
solvent or solvent mixture. It is clear that this amphiphilic
nature is found precisely in the soluble amphiphiles or surfac-
tants.

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