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CARBODIIMIDES INHIBIT THE ACID-INDUCED PURPLE-TO-BLUE TRANSITION OF BACTERIORHODOPSIN

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Summary

Reaction of purple membrane with water soluble carbodiimides inhibits the spectral transition from purple to blue observed at acid pH. The pK and Hill constant for this transition are shifted from 3.4 to 2.6 and from 1.8 to 0.85, respectively. The results suggest a connection between the uptake side of the proton pump and the purple-to-blue transition.

The purple membrane of *Halobacterium halobium* functions as an extremely simple ion pump. The molecular mechanism of bacteriorhodopsin's light-induced proton pump activity is now under intensive study (for a comprehensive review, see Ref. 1).

Early experiments by Oesterhelt and Stoeckenius showed that the purple pigment (λ_{max} = 570 nm) turns blue (λ_{max} = 605 nm) at acid pH [2]. Recent attention has been drawn to this blue form of the purple membrane because of the transient appearance of blue photo-intermediates during the bacteriorhodopsin photochemical cycle [3—7]. The apparent pK for appearance of the blue pigment is about 3, suggesting the involvement of carboxyl groups. One model for the molecular mechanism of the proton pump proposed by Lewis et al. [8] makes a direct connection between the blue photointermediate K, the protonation of a carboxyl group on the release side of the pump, and the acid-induced blue pigment. Fischer and Oesterhelt suggested that the 605 nm pigment results from the protonation of the counter-ion to the retinal Schiff base [7].

We have been studying derivatives of the purple membrane with chemically modified carboxyl groups. The purple membrane reacts with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) near neutral pH, and the product displays an altered photochemical cycle and increased steady-state release of protons at alkaline pH [9]. These alterations in photochemical activity might be due, in part, to the blocking of the carboxyl group (or groups) involved in formation of the acid-induced blue pigment. The spectrophotometric titrations reported here provide evidence for this suggestion.

Purple membranes were isolated from *H. halobium* S_9 by the method of Oesterhelt and Stoeckenius [10], and reacted with carbodiimide at pH 7.0 as previously reported [9]. Unbuffered samples of purple membrane were titrated with HCl to the desired pH with a syringe microburet, and the absorption spectrum was immediately recorded. The pH was measured before and after each spectrum and generally did not differ by more than 0.05 pH units. The entire titration from pH 7 to 2 was completed with less than 0.05 ml of acid. Samples were initially light-adapted. However, rapid dark-adaptation has been found for purple membrane at low pH [5]. Thus, our spectra probably represent a mixture of light- and dark-adapted membranes. Mowery et al. [5] have shown that both light- and dark-adapted membranes have the same pK values and Hill plot slopes for the purple-to-blue transition.

At low pH, the aggregated membranes adsorbed to glassware. As a result, the concentration of the sample decreased during the titration. In order to correct for this loss, data was plotted with reference to the absorbance at the isosbestic point at 584 nm. The fractional conversion Y_H of bacteriorhodopsin to the blue form may be calculated at a particular hydrogen ion concentration, H, as follows:

$$Y_H = \frac{R_H - R_N}{R_A - R_N}$$

where R is the ratio of absorbances A_{605}/A_{584}. The subscript N signifies the average ratio at neutral pH, while A refers to the average ratio at pH values well below the pK. For these calculations, we have ignored the contributions of the reported spectral changes between pH 3.5 and 7, and between 1.5 and 0 [5]. For the results shown in Fig. 1, two data points near pH 2.0 are off the line calculated for a simple titration curve. This could be due to the difficulty in accurately measuring R_A for the carbodiimide-treated sample, or it may reflect a real increase in the slope of the curve below pH 2.5. As a first approximation, the contribution of light scattering to the measured absorbance is proportional at a given pH to the membrane concentration. Thus, the use of absorbance ratios (over a narrow wavelength range) corrects for differences in light scattering.

The titration curves for the purple-to-blue transitions of purple membrane and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide-reacted purple membrane are shown in Fig. 1, where the fraction of the blue pigment is plotted against pH. Two significant differences are evident. The apparent pK for the purple membrane control is 3.4, while that for 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide-reacted purple membrane is 2.6. A second differ-

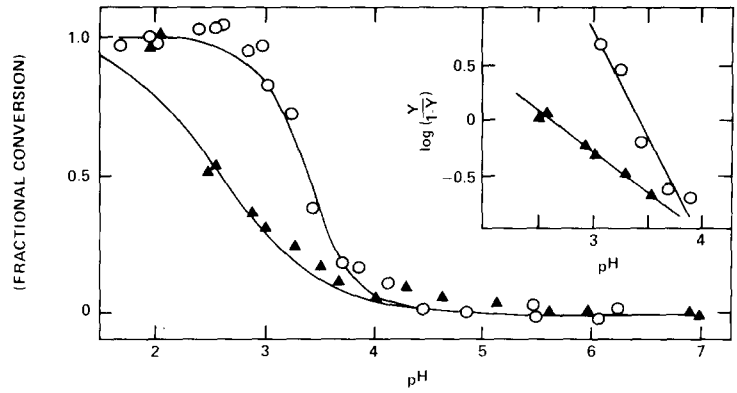

Fig. 1. Titration curves for the purple-to-blue transition. Y, mole fraction of blue pigment at a particular pH (see methods). Circles: purple membrane. Triangles: 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide-modified purple membrane. Lines were calculated for pK values of 2.6 and 3.4, with Hill constants of 1 and 2, respectively. Samples were 2.0 ml containing 10^{-5} M bacteriorhodopsin in deionized water, 23°C. Inset: Hill plot of titration data.

ence is in the slopes of the titration curves. Hill plots (Fig. 1, inset) show a slope of 1.8 for purple membrane, while the 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide-treated membrane has a slope of 0.85.

The apparent pK of 3.4 reported here for the purple-to-blue transition is close to the value of 3.2 found by Oesterhelt and Stoeckenius for purple membrane suspensions [2]. Moreover, the slope of the Hill plot of 1.8 is close to the value of 1.7 reported by Mowery et al. [5] for purple membranes in polyacrylamide gels. Therefore, the values we have found for purple membrane treated with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (a pK value nearly 1 pH unit lower, and a Hill plot with a slope close to 1) show that the purple-to-blue transition has been inhibited by chemical modification. Purple membrane reacted with 1-cyclohexylamino-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate showed the same pK shift and slope change found with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide-reacted purple membrane. The incorporation of radioactivity (Table I) indicates a minimum of one modified site per bacteriorhodopsin molecule.

One possible explanation of the carbodiimide effect is a difference in state of aggregation, since the carbodiimide reaction appears to induce aggrega-

TABLE I

STOICHIOMETRY OF CARBODIIMIDE REACTION

The reaction with (*Me*-^{3}H)-labeled CMC was done under conditions similar to the nonradioactive carbodiimide reactions: bacteriorhodopsin, $1.4 \cdot 10^{-5}$ M; NaCl, 36 mM; CMC, 2.9 mM. Two samples were prepared: A contained (*Me*-^{3}H)-labeled CMC ($6.9 \cdot 10^{10}$ cpm per mol) and B contained non-radioactive CMC. After 5 h reaction on ice, the samples were centrifuged at 40 000 × *g* for 30 min at 5°C. The pellet of B was resuspended in radioactive CMC and then immediately centrifuged. (This permitted a correction for non-covalent CMC binding to purple membrane.) Both samples were then washed with NaCl as previously described [9]. Radioactivity was measured by liquid scintillation counting in Aquasol (New England Nuclear). CMC, 1-cyclohexylamino-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate. BR, bacteriorhodopsin.

Sample	cpm	nmol CMC	nmol BR	Molar ratio CMC/BR
A. Purple membrane	2251	32.7	18.1	1.8
B. Purple membrane pre-reacted with CMC	564	8.7	17.7	0.5

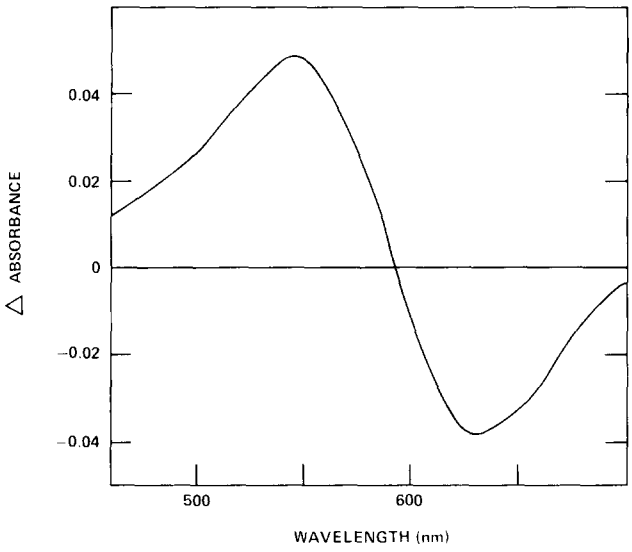

Fig. 2. Difference spectrum at pH 3.07 of polyacrylamide gels containing purple membrane, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide-modified membrane minus unmodified membrane. Gels were 5.5% acrylamide, 0.15% bis acrylamide, $1.5 \cdot 10^{-5}$ M bacteriorhodopsin, 2 mm thick. After polymerization, one gel was soaked in 0.06 M 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, pH 7.0 for 5 h at 0° C. Both were subsequently washed for 24 h at 0° C with several changes of ice-cold 0.05 M NaCl and then equilibrated with 0.05 M phthalate buffer, pH 3.07, for 24 h. Gels were mounted at 45° angles to the incident light beam.

tion of the membranes. In view of this, we reacted 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide with purple membrane fragments that were immobilized in polyacrylamide gels and therefore prevented from aggregating [5]. A difference spectrum for carbodiimide reacted versus unreacted purple membrane in gels is shown in Fig. 2 for pH 3.07. The inhibition of the formation of the blue pigment is clearly evident in gels and thus it cannot be due to aggregation.

The steep slope of the purple-to-blue transition in unreacted purple membrane is typical of the titration of cooperatively interacting groups. The diminished slope of the transition after carbodiimide treatment shows that the cooperativity has been abolished. The loss of cooperativity could be due to a large-scale decrease in the negative surface charge of the membrane. However, we have previously shown that carbodiimide treatment does not result in such changes [9]. It is more likely that a cluster of several carboxyl groups interact in the purple-to-blue transition. Thus, carbodiimides block one group in this cluster, abolishing the cooperative interaction and inhibiting the formation of blue pigment. We have previously reported evidence that the carbodiimide reaction inhibits the uptake side of the proton pump [9]. The present results suggest the possibility that proton uptake and the purple-to-blue transition are connected, since the same reaction seems to inhibit both.

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