Transient deprotonation of bacterial halorhodopsin by photoexcited base

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The excited singlet state of 6-methoxyquinoline is basic enough to abstract a proton from water and generate OH^-. Pulse excitation of such a solution undergoes a massive transient alkalinization. This procedure was employed to monitor the deprotonation of the Schiff base of halorhodopsin. Both the deprotonation and reprotonation of the chromophore are diffusion controlled reactions.

Halorhodopsin *Difffusion-controlled reaction* *Excited state proton transfer* *pH jump* *6-Methoxyquinoline*

1. INTRODUCTION

The pK shift of heterocyclic compounds, which follows their excitation to the first electronic singlet state [1], is utilized to generate a massive synchronized OH^- pulse in aqueous solution.

The pK of 6-methoxyquinoline increases upon excitation from 4.95 of the ground state to 11.9 of the excited molecule. Such a basic compound can abstract a proton from the surrounding water to produce an ion pair $\phi N^*H^+ + OH^-$. Thus the synchronized excitation of aqueous solution of 6-methoxyquinoline can be an effective OH^- generator. This compound can be used for pulse alkalinization of solution, in analogy with the laser induced proton pulse technique [2].

In this communication we shall demonstrate the various proton transfer reactions following excitation of 6-methoxyquinoline. The applicability of this methodology for pulse deprotonation of halorhodopsin will also be demonstrated.

2. MATERIALS AND METHODS

6-Methoxyquinoline, Aldrich Chemical Company Lot 2329 HL, was used without further purification.

Halorhodopsin from *H. halobium* was a generous gift of Dr J. Lanyi and Dr B. Schobert, Physiology and Biophysics, University of California, Irvine, CA 92717. Before use the protein was dialyzed for 24 h (4°C) against 4 M NaCl (1000-fold volume).

The pH indicators (or the protein) were placed with 2 mM 6-methoxyquinoline in a 4 face cuvette 10×3 mm. A molectron UV 14 nitrogen laser was used for excitation. The exciting pulse was focused to a rectangle 1.5 mm high covering the full length of the cuvette (10 mm). The monitoring beam of a HeNe laser (633 nm) 1 mm in diameter was passed through the excited space in the cuvette, perpendicular to the excitation pulse. This configuration ensures that the monitored space is homogeneous with respect to the photochemical reactions. The content of the cuvette is constantly mixed by a small magnetic fly.

The probing beam was directed through a monochromator to an EMI 9684B photomultiplier. The signals were recorded by a Biomation 8100 transient recorder and accumulated by a Nicollet 1170 signal averager.

3. RESULTS AND DISCUSSION

3.1. *Alkalinization pulse*

The pK of the ground state of 6-methoxyquinoline (pK_o) is 4.95. Still, the emission of this compound at pH 9.5 is mostly that of the excited pro-

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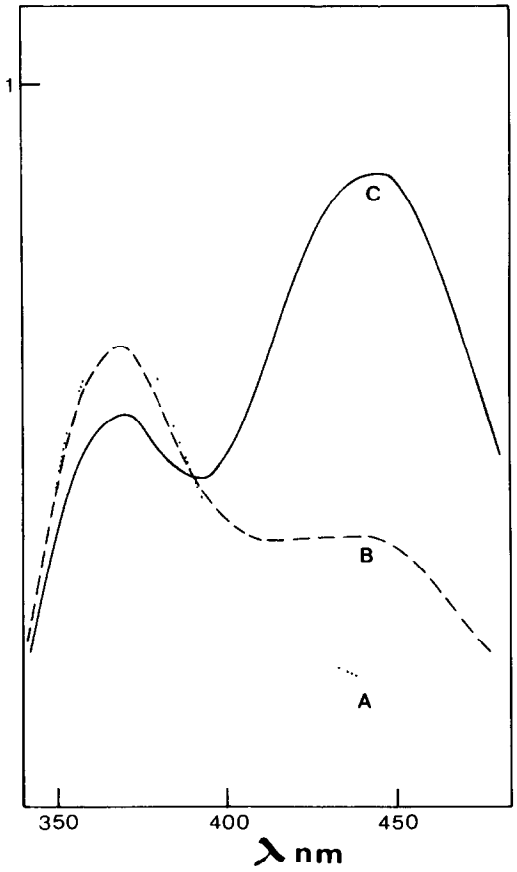
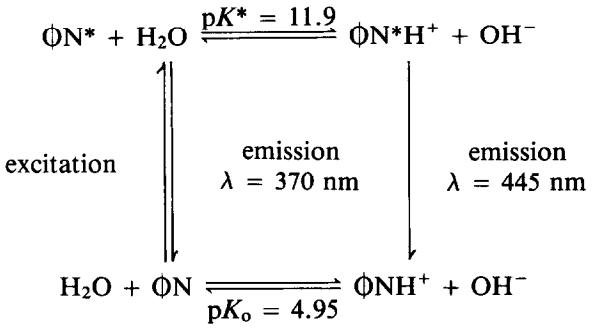


Fig.1. Fluorescence spectrum of 10 μM 6-methoxyquinoline in H_2O excitation at 328 nm. Line A, pH 12.7; line B, pH 11.6; line C, pH 9.6. Ordinate: fluorescence intensity in arbitrary units.

tonated state ((ϕN^*H^+), λ_{max} = 445 mm) (fig.1). As at this pH the ground state population is exclusively of ϕN, the ϕN^*H^+ must be formed by a sequence of events shown in scheme 1.



Scheme 1.

The lifetime of ϕN^* is 1.6 ns. Within this time frame a collision of ϕN^* with H^+ (at pH 9.5) is very improbable. Thus we conclude that the formation of ϕN^*H^+ is through proton abstraction from water.

Detailed kinetic analysis of time resolved fluorescence of 6-methoxyquinoline in aqueous solution indicates that the time constant of ϕN^*H^+ formation is 3 ns [3].

The capacity of 6-methoxyquinoline to function as OH^- generator and alter pH of aqueous solutions is demonstrated in fig.2. As seen in the figure, following the laser pulse, OH^- was generated and, upon collision with indicator molecules, deprotonated them. The short transient is in agreement with the diffusion controlled nature of the reaction. The time constant of deprotonation is τ = 150 ns while the whole event relaxes to prepulse state with τ = 2.75 μs. The magnitude of the initial OH^- pulse can be estimated from the initial velocity of the reaction (13 m/s), the protonated indicator concentration (0.37 mM) and the rate constant of the reaction (k = 10^{10} $M^{-1}\cdot s^{-1}$ [4]). About 8 μM OH^- was generated by each laser pulse. Thus 6-methoxyquinoline can be used as an effective agent for pulse alkalinization of aqueous solutions.

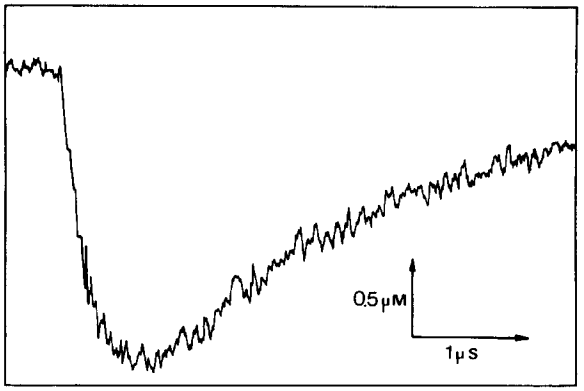


Fig.2. Transient deprotonation dynamics of bromothymol blue (500 μM) in the presence of 2 mM 6-methoxyquinoline, at pH 6.5. The sample was irradiated by nitrogen laser pulse (5 mJ, 10 ns width at half maximum). The transient absorbance was monitored at 633 nm using an HeNe CW laser. The transient is a sum of 1024 events accumulated at a repetition rate of 15 Hz.

3.2. *Acidification pulse*

The lifetime of ϕN^*H^+ formed during the laser pulse is $\tau = 19.5 \pm 0.5$ ns [3]. Thus within 50 ns after the pulse all of the ϕN^*H^+ population decays to the ground state species ϕNH^+. The deprotonation of ϕNH^+ can follow 2 mechanisms: proton dissociation and collisional proton transfer. Of the two pathways the former is inherently slow. A compound with $pK = 5$ will discharge its proton with a time constant of $\tau = 10\ \mu s$. Within this time frame collisional proton transfer is a very effective mechanism for deprotonation. The proton acceptors in the collisional pathway are both OH^- (generated by the pulse) and indicator (In^-) present in the solution. The 2 compounds compete for ϕNH^+ but only the collisions between ϕNH^+ and In^- are observed. This reaction is demonstrated in fig. 3A and B. Frame A documents the acidification of bromothymol blue ($pK = 7.0$), at pH 8.2, while in frame B we present kinetics measured with bromocresol green, at pH 6.4, i.e. 1.4 pH units above its pK. In both cases the indicator serves as efficient acceptor through the collisional proton transfer.

The dual nature of the laser induced pH jump causing both alkalinization and protonation of different compounds was known before [5]. Yet the long lifetime of ϕNH^+ amplifies the capacity for monitoring the acidification pulse which proceeds simultaneously with the increased OH^- concentration. The understanding of the mechanism of this reaction not only clarifies the apparent paradox, but also allows us to select, through the prepulse pH, what will be the dominating event after the pulse, an advantage which we exploited for our studies with halorhodopsin.

3.3. *Deprotonated cycle of halorhodopsin*

Halorhodopsin ($pK = 8.9$ at 4 M NaCl) is stable in its protonated Schiff base form ($HR\text{-}H^+$). Deprotonation destabilizes the protein and the retinal is slowly released [5]. Thus we decided to use the 6-methoxyquinoline as OH^- generator and to follow the deprotonation of $HR\text{-}H^+$. For this purpose we carried out the experiment at pH 8.2–8.5. Under these conditions the chromophore is still protonated while all amino acid residues (excluding lysine and arginine) are in their basic form. This initial state ensures that the perturbation will cause a deprotonation of the Schiff base

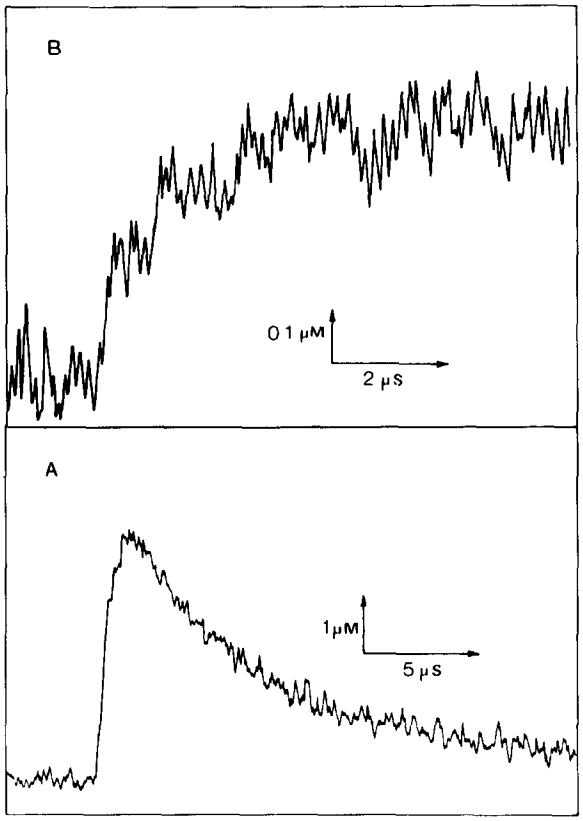


Fig.3. Transient protonation of bromothymol blue (A) and bromocresol green (B) by ground state 6-methoxyquinolinium. The solution of each dye (50 μM) plus 2 mM 6-methoxyquinoline was photoexcited at a pH higher than the pK of the indicator. The dynamics of bromothymol blue was measured, at pH 8.3 ($pK = 7.0$) and bromocresol green, at pH 6.4 ($pK = 4.95$). Experimental conditions as in fig.2.

(monitored at 633 nm), while protonation (by ϕNH^+) of any side chains will have no contribution at the monitoring wavelength.

As seen in fig.4A, excitation by laser led to rapid deprotonation of the chromophore. The amount of halorhodopsin which was deprotonated by each pulse was nearly 10% of the total. The deprotonated state relaxes to the equilibrium level with a time constant of ~3 ms (fig.4B). Considering the concentration of the reactants ($H^+ = 10^{-8}$ M; HR (deprotonated) = 1.10^{-6} M) we estimate that the rate of protonation ($HR + H^+ \longrightarrow HR \cdot H^+$) is $10^9\ M^{-1} \cdot s^{-1}$. This rate constant corresponds to a diffusion controlled reaction.

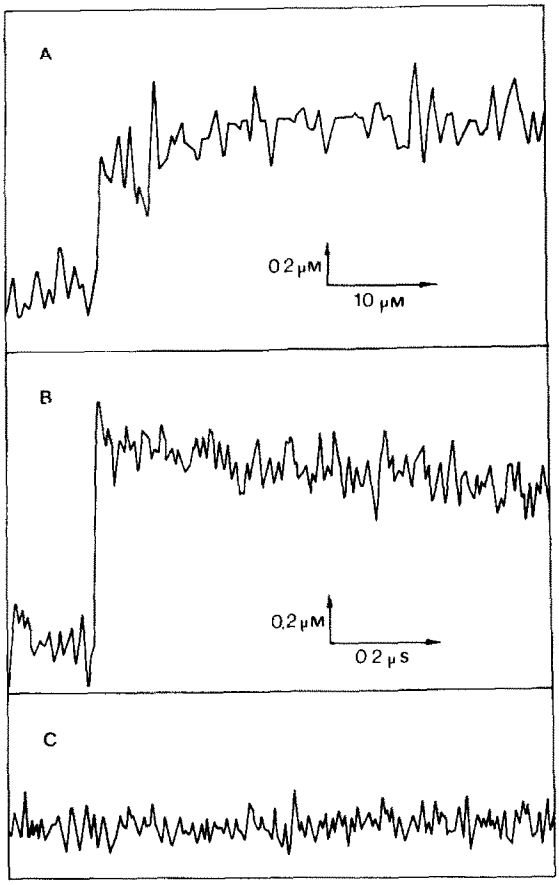


Fig.4. Transient absorption of halorhodopsin following alkalinization pulse by 6-methoxyquinoline. Halorhodopsin, 10.5 μM in 4 M NaCl plus 2 mM 6-methoxyquinoline, pH 8.5, was excited by nitrogen laser pulses and the deprotonation was monitored at 633 nm (ϵ_{633} = 15000 $M^{-1} \cdot cm^{-1}$). The signal is a sum of 4098 events accumulated at a repetition rate of 15 Hz. Upward deflection corresponds with the bleaching of the 578 nm band of the protonated Schiff base. A, dynamics measured at 100 ns/address resolution. B, measurements at 2 μs/address resolution. C, control: halorhodopsin in the absence of 6-methoxyquinoline.

It is of interest to point out that the deprotonated schiff base can be formed either at alkaline pH or by photodeprotonation [6]. The protonation of HR formed by photobleaching is extremely slow (hours) [5] while that generated by an alkalinization jump is too fast to be resolved by stopped flow spectrophotometry [7]. The rapid reprotonation we observed indicates that the species we monitor are not identical with the photoproduct. What is more, the diffusion controlled rate of the reaction supports the conclusion [6] that the Schiff base in halorhodopsin is located close to the protein surface.

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REFERENCES

[1] Ireland, J.R. and Wyatt, P.A. (1976) Adv. Phys. Org. Chem. 12, 131–221.
[2] Gutman, M. (1984) Methods Biochem. Anal. 30, 1–103.
[3] Huppert, D. and Gutman, M., unpublished.
[4] Eigen, M. (1964) Ang. Chemi. Int. Edn 3, 1–19.
[5] Gutman, M., Nachliel, E., Gershon, E., Giniger, R. and Pines, E. (1983) J. Am. Chem. Soc. 105, 2210–2216.
[6] Lanyi, J.K. (1985) Annu. Rev. Biophys., in press.
[7] Lanyi, J.K. and Schobert, B. (1983) Biochemistry 22, 2763–2769.
[8] Lanyi, J.K. and Gutman, M., unpublished.