Inhibition of the M1 → M2 ($M_{closed} \rightarrow M_{open}$) transition in the D96N mutant photocycle and its relation to the corresponding transition in wild-type bacteriorhodopsin

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Received 1 April 1997

Abstract Glutaraldehyde, lutetium ions and glycerol inhibit the blue shift of the difference spectra maximum of the M intermediate in the D96N mutant. The M formed has a spectrum indistinguishable from the M intermediate in wild-type bacteriorhodopsin. It has been concluded that the M_{open} form previously described by us is identical to the M2 and M_n intermediates postulated by Zimanyi et al. (*Photochem. Photobiol.* (1992) 56, 1049–1055) and Sasaki et al. (*J. Biol. Chem.* (1992) 267, 20782–20786), respectively. It is supposed that its formation is accompanied by the appearance of the cytoplasmic proton half-channel. M_{open} in the wild-type protein is present in a very low amount due to the shift of the $M_{closed} \leftrightarrow M_{open}$ equilibrium towards the M_{closed}. The inhibitors used do not prevent the multiphase pattern of the M formation in either mutant or wild-type proteins.

Key words: Bacteriorhodopsin; Photocycle; Proton transport; Azide; Purple membrane; D96N mutant; *Halobacterium salinarium*

1. Introduction

It is well known that the M form, the central intermediate of the bR photocycle, in fact comprises a set of different forms with similar absorption spectra [1–9]. One of the most intensely discussed problems is the transition between the two M forms called M1 and M2. It is supposed that the transition is associated with the reprotonation switch that changes access of the Schiff base from the extracellular side to the cytoplasmic side. The scheme L ↔ M1 → M2 was originally proposed to explain the complicated kinetics of the M intermediate formation [1,2,4]. Recently, it was found that in both the D96N mutant and other mutants where Asp-96 is changed as a second residue replacement, the two M states have different maxima. M1 has the maximum of M in the wild-type bR, while the maximum of late M is significantly blue-shifted (404 nm) [5,6,9]. When studying the action of glutaraldehyde, lutetium ions, glycerol and sucrose on the azide-facilitated M decay in the D96N mutant [10,11], we came to the conclusion that there are two M forms (M_{closed} and M_{open}) differing in the accessibility of the Schiff base for azide and, probably, also for water molecules.

In the present paper, we used the above-mentioned inhibitors to investigate the spectra of the M_{closed} and M_{open} in the D96N mutant. It has been supposed that the M_{open} form corresponds to the M2 form and to the M_n intermediate described by Sasaki et al. [12]. It is found that the $M_{closed} \leftrightarrow M_{open}$ (M1 ↔ M2) equilibrium is shifted to the M_{open} state in the D96N mutant. As for the wild-type bR, the equilibrium is strongly shifted to the M_{closed} form, and the M_{open} form cannot be revealed. Moreover, inhibition of the M_{open} (M2) formation does not prevent the multiphase pattern of the M formation both in the D96N mutant and wild-type bR. This is opposite to what would be expected from the kinetic scheme of Varo and Lanyi [1,2,4].

2. Materials and methods

All measurements were carried out in freshly prepared purple membrane sheets from the halobacterial wild-type ET1001 and D96N mutant strain. The latter was kindly donated by Prof. D. Oesterhelt (Max-Planck Institut für Biochimie, Germany).

Measurements were performed on the light-adapted purple membrane suspension at 20°C. The bR photocycle transient absorbance changes of bR were measured using a laboratory-built single-beam spectrophotometer as previously described [6,10,11]. The time-resolved difference spectra were obtained by computer processing of 25 curves of absorption change signals measured at 320–390 and 420–490 nm intervals with a 10 nm step and at 390–420 nm interval with a 3 nm step. Light flashes were provided by a frequency-doubled Quantel Nd-YAG-481 laser (wavelength, 532 nm; pulse half-width, 15 ns; energy, 10 mJ). The time-resolved absorbance change curves were decomposed into components with the aid of the DISCRETE program written by S. Provencher.

Glutaraldehyde treatment of bR was performed as previously described [6,11].

3. Results and discussion

It is known that the maximum in the difference spectrum of the M intermediate in the D96N mutant is at 404 nm [5,6,9], whereas the maximum of the M intermediate in the wild-type bR is located at 412 nm (Fig. 1A). Comparison of the time-resolved difference spectra of the M intermediate measured at different time intervals after an exciting laser flash reveals some blue shift of the maximum for the D96N mutant during the M formation (Fig. 2A). The shift is absent in the case of the M formation in the wild-type bR (Fig. 2B). It was shown by Zimanyi et al. [5] that the blue shift of the maximum of the M intermediate of the D96N mutant is particularly distinct at high pH value due to significant acceleration of the fast phase of the M intermediate formation. The spectra of early M form(s) of the mutant is analogous to the M spectra of the wild-type bR.

Glutaraldehyde, lutetium ions and glycerol which inhibit the M decay in the wild-type bR photocycle do not affect either the spectrum of its M intermediate (Fig. 1B–D) or the

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Abbreviations: bR, bacteriorhodopsin; GA, glutaraldehyde; PM, purple membrane

PII S0014-5793(97)00474-2

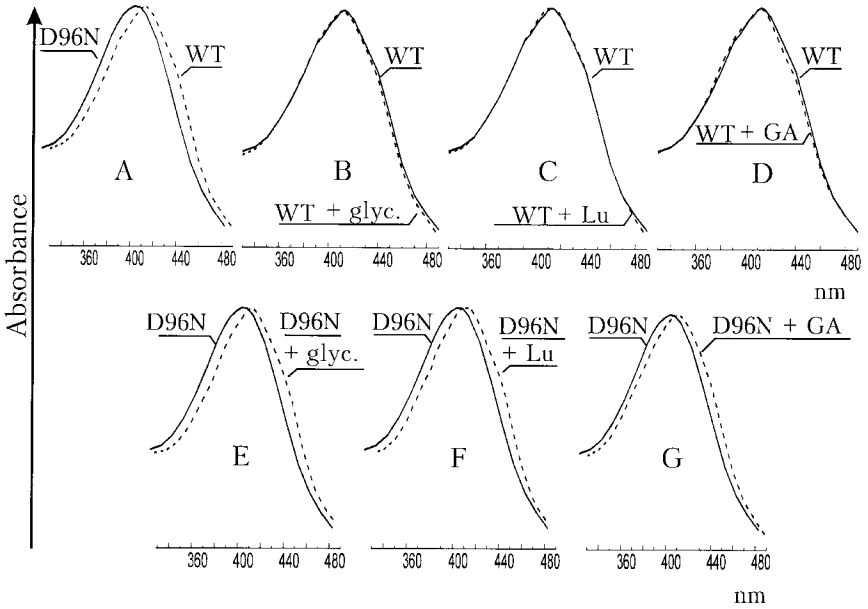

Fig. 1. Effects of the inhibitors of the $M_{closed} \rightarrow M_{open}$ transition on the difference spectra of the M intermediate. The spectra for the D96N mutant were measured in 3 ms after the exciting flash. The spectra for the untreated wild-type bR (WT) were measured in 0.6 ms after the exciting flash. The spectra for the treated wild-type bR were measured in 3 ms after the exciting flash. To facilitate the comparison, all the spectra are presented with the equal magnitudes. A: Comparison of the M spectra for the D96N mutant and for the wild-type bR. B–D: Effects of the 83% glycerol, 5 mM $LuCl_3$ and glutaraldehyde, respectively, on the M spectra of the wild-type bR. E–G: Effects of the 83% glycerol, 5 mM $LuCl_3$ and glutaraldehyde, respectively, on the M spectra of the D96N mutant. The assay medium was: A,C,D,F,G – 1 M NaCl, 10 mM HEPES (pH 7); B,E – 10 mM HEPES (pH 7).

amplitude of the flash-induced optical changes at 400 nm. The agents also strongly inhibit the azide-facilitated M decay and induce the red shift of the differential spectra of the D96N mutant (Fig. 1E–G). This shift is similar to the shift between spectra of the M intermediates of the untreated D96N mutant and wild-type bR (Fig. 1A) and is related to the ability of all the agents to prevent the blue shift of the M intermediate spectra developing during its formation (Fig. 2C). The inhibitors induce some decrease (by 10–20%) of the amplitude of the optical changes at 400 nm.

In our previous paper, on the basis of inhibitory analysis of glutaraldehyde, lutetium ions, glycerol and sucrose action on the azide-facilitated M decay in the D96N mutant [6], we concluded that two M forms (M_{closed} and M_{open}) exist. These forms are in equilibrium and differ in the accessibility of the Schiff base for azide and probably, also for water molecules. The inhibitors shift the equilibrium toward the M_{closed} form. Lutetium ions (5 mM) and glycerol (83%) decrease the M_{open} concentration by more than 2 orders. The results obtained in the present work indicate that whereas M_{closed} has a maximum at 412 nm, the spectrum of the M_{open} is blue shifted and located at 404 nm or a shorter wavelength because there is no assurance that the equilibrium is shifted fully to the M_{open} state in untreated mutant protein. We supposed earlier [6,11] that the $M_{closed} \rightarrow M_{open}$ transition is a result of the appearance of a cleft in bR on the cytoplasmic side of the protein. This supposition is in accordance with the modern conception of the mechanism of the Schiff base reprotonation during the bR photocycle [13–18]. We believe that this water cleft is permeable for azide and thus facilitates the reprotonation of the Schiff base by artificial proton donors. Spectral identification indicates that M_{open} is equivalent to the M2 form. Thus, in terms of the M1 → M2 transition the reprotonation switch is the opening of the water cleft, i.e. the appearance of the cytoplasmic proton half-channel. The cleft originally postulated by Skulachev [13,14] was then directly proved by elec-

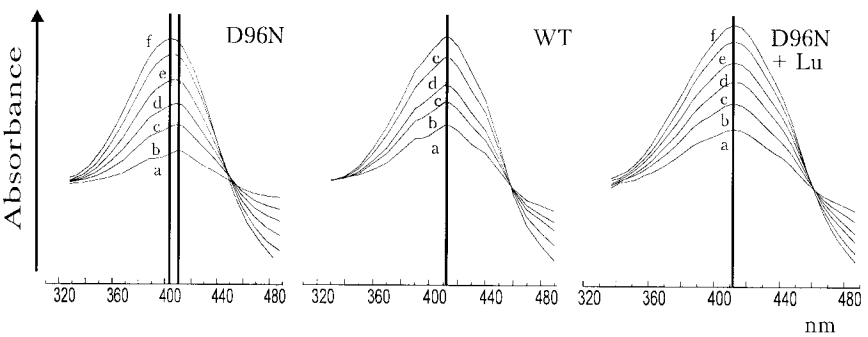

Fig. 2. Time-resolved difference spectra of the M intermediate. A: Untreated D96N mutant. B: Untreated wild-type bR (WT). C: D96N treated within 5 mM $LuCl_3$. A: Spectra measured in (a) 0.05, (b) 0.11, (c) 0.2, (d) 0.4, (e) 1, (f) 11 ms after the exciting flash. B: Spectra measured in (a) 0.08, (b) 0.11, (c) 0.2, (d) 0.38, and (e) 0.7 ms after the exciting flash. C: Spectra measured in (a) 0.05, (b) 0.11, (c) 0.21, (d) 0.4, (e) 1, and (f) 11 ms after the exciting flash. The assay medium was: A,B, 10 mM HEPES (pH 7); C, 10 mM HEPES (pH 7), 83% glycerol.

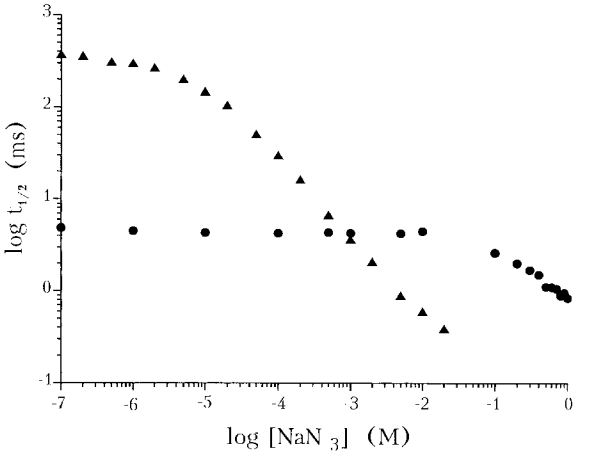

Fig. 3. Dependence of the M decay in the D96N mutant (▲) and in the wild-type bR (●) on the azide concentration. The assay medium was: 1 M NaCl (pH 5), 10 mM Na-citrate, 10 μM bR.

tron diffraction data on the structure of the M intermediate in the D96G mutant [19]. In M, a tilt of the cytoplasmic part of the helix F in comparison with the initial bR state has been shown. A similar difference between the conformation of the initial bR state and the N intermediate was described in the X-ray [20] and electron [21] diffraction studies. The Fourier transform infrared spectroscopy data [12] revealed similarity of the protein conformation in the N intermediate and in the long-living M intermediate (denoted as M_n) in the D96N mutant. We did not find any indications of existence of the M forms other than the M_{open} intermediate even at very high pH values. Thus, M_{open} seems to be identical not only to M2 but also to M_n.

Within the framework of the $L \leftrightarrow M1 \rightarrow M2$ scheme, it is usually assumed that the wild-type bR transforms completely into the M2 during the M formation. The blue shifted spectra of the M2 form of the D96N mutant in comparison with the analogous M2 form of the wild-type bR is attributed by Zimanyi et al. [5] to a difference in the interaction of the Schiff base with the Asn-96 and with Asp-96. Our interpretation of the blue shift is quite different. First of all, the M form in the wild-type does not correspond to the M2 (M_{open}) of the D96N mutant and is similar to the M_{closed} state. Fig. 3 shows the comparison of the acceleration of the M decay by azide in the D96N mutant and in the wild-type bR. Note that 2 order higher concentrations of the azide are necessary to achieve the same rates of M decay in the wild-type protein (see also [10,11,22]). In this respect, the wild-type bR resembles the inhibitor-treated D96N mutant [10,11]. We suppose that this difference is due to the different concentrations of the M_{open} state in the D96N mutant and the wild-type bR rather than to the difference in the sterical hindrances. The concentration of the M_{open} during the photocycle in the wild-type bR must also be small for another reason. This M form has the cleft and formation of the cleft should lead to the immediate lowering of the pK of the Asp-96 and protonation of the Schiff base [15–17]. Thus, this form in the photocycle of the wild-type bR limits the rate of the $M \rightarrow N$ transition and cannot be identified for a kinetic reason. We believe that the spectra of the M2 might also be blue shifted similarly to M2 in the D96N mutant. The reason for the shift is probably related to the appearance of water molecules near the Schiff base, and thus to the increase in the dielectric constant. Electron diffraction data confirm this idea. Significant structural changes described for the D96G mutant were not found for the wild-type bR [19–21,23–26]. Moreover, it is highly improbable that this M_{open} can be stabilized in an amount sufficient for electron or X-ray diffraction analysis. The shift of the equilibrium between $M_{closed} \leftrightarrow M_{open}$ towards M_{open} seems to be an intrinsic feature of the Asp-96 → Asn replacement. Some other mutants (D96G, D96A) are not so sensitive to azide [15,22,27]. We suppose that the concentration of M_{open} in these mutants is lower than in the D96N mutant. Moreover, our model predicts that the spectra of the M intermediate would be blue shifted to a lesser extent in comparison to the D96N mutant.

The scheme $L \leftrightarrow M1 \rightarrow M2$ was originally proposed to explain the complicated character of the kinetics of the M intermediate formation [1,2,4]. We have made an attempt to verify this, using the inhibitors of the $M1 \rightarrow M2$ transition (Table 1). In spite of the fact that all the inhibitors used decrease the equilibrium concentration of M_{open} by 2–3 orders, none of them abolishes the multiphase kinetics of the M formation in either the D96N mutant or the wild-type bR (note, that the fastest component reflects the $K \rightarrow L$ transition and that glycerol eliminates this component due to its effect [4,6]). Thus, multiphase formation of the M intermediate could be explained by the presence of different conformational states in bR [6,28,29], or by the consecutive formation of different M forms belonging to the M_{closed} pool (in the latter, the similar scheme $L \leftrightarrow M' \leftrightarrow M'' \ldots \leftrightarrow M^i$ may be valid, but all these M states seem to belong to the M_{closed} pool). The rate of

Table 1
Kinetic parameters of the M intermediate formation wild-type bR (ET 1001)

Kinetic phase	Untreated PM		PM in 2 mM $LuCl_3$		Glutaraldehyde-treated PM		PM in 80% glycerol	
	1 M NaCl (pH 7) (τ, μS)	(%)	1 M NaCl (pH 7) (τ, μS)	(%)	1 M NaCl (pH 5) (τ, μS)	(%)	(pH 5) (τ, μS)	(%)
1	2	10	1	10	2	10	–	–
2	60	55	55	30	50	50	14	24
3	250	35	200	40	220	40	70	53
4	–	–	800	20	–	–	510	22
D96N mutant								
1	1	10	2	11	2	10	–	–
2	55	23	50	26	60	50	8	17
3	220	42	200	45	220	30	50	60
4	1000	25	1300	18	1300	10	400	24

the $M_{closed}(M1) \leftrightarrow M_{open}(M2)$ equilibration is a matter of future experiments. In any case, it is obvious that it cannot be deduced from the kinetics of the M formation on the basis of the simple suppositions. As far as we know, this is a fast process, taking place in time scale of several microseconds. This point of view is based mainly on the ability of azide at high concentrations to accelerate the M decay to the microsecond time domain (for details, see [6,10,11,30]). Perhaps, in some cases (for instance, for some double mutants with the very distinct phases of the M formation [9] or at very high pH values [5]), the slower phase reflects the $M1 \rightarrow M2$ transition, but this is not the case for the wild-type bR and the D96N mutant at neutral and low pH values. Thus, our data suggest that the kinetic parameters cannot be used for evaluation of the thermodynamic characteristic as well as elementary constants of the $M1 \leftrightarrow M2$ transition in wild-type bR and the D96N mutant.

Acknowledgements: This work was supported by the Russian Foundation for Basic Research (Grant 97-04-49749) and INTAS (Grant 93-2852).

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