Diamines reverse the direction on the bacteriorhodopsin proton pump

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Purple membranes oriented and immobilized in gel were soaked in solutions containing different monoamines and diamines. The flash-excited electric signals showed a reversal of the direction of the charge motion in bacteriorhodopsin in the case of diamines which was interpreted as the reversal of the proton pump. Monoamines had negligible influence on the electric signals. The influence of tetramethylethylenediamine was also studied in detail.

Bacteriorhodopsin *Proton pump* *Photoelectric signal* *Orientation* *Membrane fragment* *Tetramethylethylenediamine*

1. INTRODUCTION

It is well established that bacteriorhodopsin (bR) molecules in *Halobacterium halobium* pump protons under light irradiation from the inside to the outside of the cells [1]. The proton translocation activity is preserved in purple membranes (pm-s) isolated from the bacteria. The flash-induced electric signals recorded in oriented pm-s correspond to the motion of charges inside the bR molecules [2,3]. The first very fast component [4] and the second microsecond component of the electric signal are negative (related to the direction of proton pumping) and are followed by 3 positive components of different lifetimes. The sum of the areas under the electric signals is not zero but a positive value. This fact is considered as a direct demonstration of a net charge translocation. Though the positive area of the electric signal may be caused by a backward moving negative charge (i.e. OH^-) we may assume safely that the electric signal corresponds to a forward moving positive charge, a proton which is generally accepted as the pumped ion by bR.

Here we report that diamines added to a solution containing bR can transform the positive integrated area of the electric signals into a negative value which we interpret as a reversal of the direction of the proton pump. A more detailed investigation of the effect of *N,N,N',N'*-tetramethylethylenediamine (TEMED) revealed several important features: the reversal can essentially be complete under certain conditions, is concentration dependent and can be abolished by adding $CaCl_2$ or NaCl to the solution.

2. MATERIALS AND METHODS

The pm-s used in the experiments were obtained from *H. halobium* strain ET 1001 using a standard procedure [5]. The concentration of bR was measured spectroscopically using an extinction coefficient of 63000 $mol^{-1} \cdot l \cdot cm^{-1}$. Orientation was achieved by the application of a d.c. electric field and immobilization in a gel [6]. The gels were cut into 0.8 × 0.3 × 0.2 cm pieces and soaked for a day in a solution (~150 ml)· containing the diamines: TEMED from Serva, *N,N*-dimeth-

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ylethylenediamine, 1,3-diaminopropane both from Aldrich and ethylenediamine from Reanal, Hungary. Ethanolamine (Ferak, Berlin) and diethylamine (Angarskii Zavod Chimreaktivov) were also used as control substances. In the diamine concentration ranges used, the pH was always between 6 and 8.

The gel pieces were put into a cuvette of 2 × 0.2 cm internal cross-section between 2 Pt electrodes separated by a distance of 1.6 cm and filled up to ~0.4 cm with their soaking liquor. The photocycle was initiated by a frequency-doubled NdYAG laser (λ = 530 nm) of ~6 mJ. The resulting electric signals which were picked up by the Pt electrodes were amplified by a home-made amplifier of 1 MHz bandwidth and processed by a transient recorder (product of the Central Research Institute of Physics, Hungary; smallest conversion time 0.1 μs, conversion range 256 quanta/channel, 1024 channels).

A few measurements were performed on pm-s in suspension. In this case, TEMED was used, and the measuring procedure was the same as in [7].

The photocycle was checked by measuring the flash-induced absorbance changes at λ = 400 and 650 nm with a photodiode. The light source was a tungsten lamp of 150 W; the wavelengths were selected by interference filters (Karl Zeiss, Jena). The data were recorded by the above system.

3. RESULTS AND DISCUSSION

Flash-excited electric signals were recorded for gel samples soaked in solutions containing each of the 6 compounds. The general behaviour with increasing concentrations of diamines was that the area under the electric signals was changed from positive to negative and at higher concentrations again to positive. The 2 amines, on the other hand, did not influence the electric signals markedly. Here we detail the investigations performed in the presence of TEMED.

TEMED is the usual polymerizing agent of acrylamide. The gel samples were washed carefully with distilled water until the electric signals were the same as in suspension [6,7]. Fig.1a shows these data. The results of the light absorption changes are shown in fig.2a–c.

As discussed in [2,3,6,7] the electric signals can be assigned to different transitions using the correspondence of their lifetimes with the lifetimes of the absorption signals. The first negative signal of fig.1a is assigned to the bR–K transition, the second small negative component (which is not resolved here) to the K–L transition. The first positive component assigned to the L–M transition means a forward motion of the proton (according to our assumption) while the long-lived positive component can be decomposed into a component decaying by the lifetime of the M–O transition and another component following the time course of the O component.

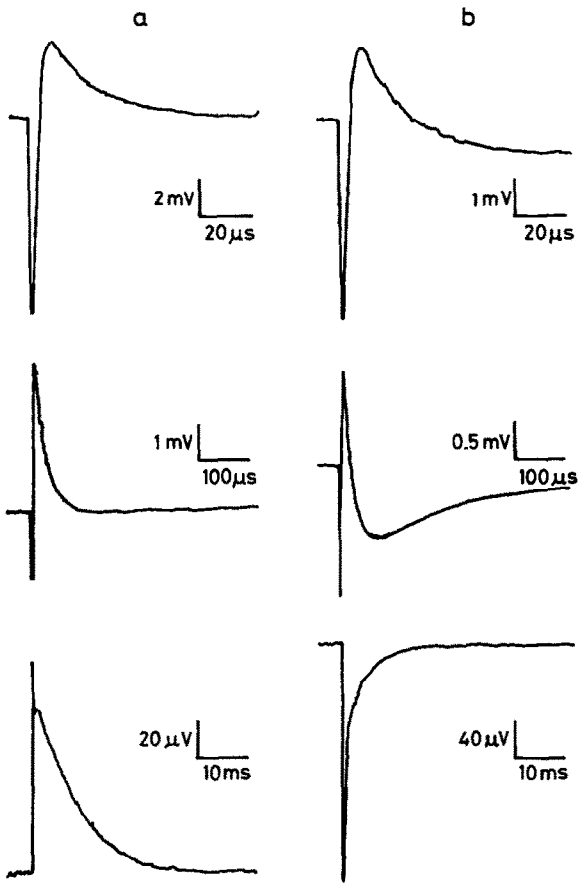

Fig.1. Electric response signals of pm-s oriented and immobilized in an acrylamide gel; bR concentration 90 μM. (a) Untreated pm-s, pH 6; (b) TEMED concentration 44 μM, pH 6.9.

TEMED, even in very low concentrations, changed the shape of the electric signals while the light absorption signals (fig.2d–f) remained practically unchanged. In fact, with a gel containing

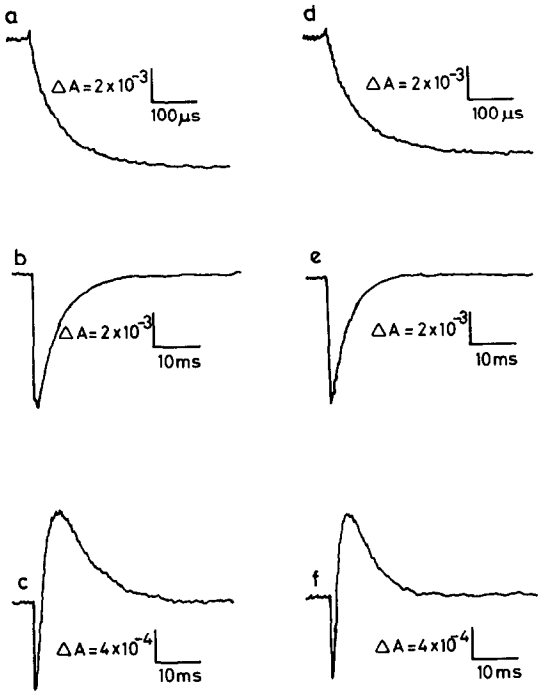

Fig.2. Light absorption changes during the bR photocycle. (a,b,d,e) λ = 400 nm, (c,f) λ = 650 nm, (a–c) untreated pm-s, (d–f) pm-s modified by TEMED.

90 μM bR, ~50 μM TEMED caused a complete reversal of the direction of proton translocation (fig.1b). It may be seen that the first negative signal is unchanged while the first positive signal, corresponding to the L–M transition in untreated pm-s, is apparently shortened in time because of the build up of a negative phase.

The negative phase has 2 components: a fast decay (τ_F ~ 300 μs) which has no partner among the absorption signals and a slower one (τ_S ~ 7 ms corresponding to the M–O transition). Both signals are assigned to occur after the formation of the M state which means that their time course is described by

$$V_F(t) = F(e^{-t/\tau_L} - e^{-t/\tau_F}) \quad (1)$$

$$V_S(t) = S(e^{-t/\tau_L} - e^{-t/\tau_S}) \quad (2)$$

Here F and S are the maximal amplitudes of the signals, and τ_L the lifetime of the L–M transition. It is easy to see that these negative voltage forms apparently shorten the lifetime of the electric signal corresponding to the L–M transition.

Similar data were registered in a broad concentration range of TEMED of the bathing solution for 2 gel samples containing 90 and 360 μM bR, respectively. The areas of all positive and negative components (A_i) were determined, summed up and normalized to the first negative signal (which is proportional to the number of excited molecules). In fig.3 the ratio $R = -\Sigma A_i/A_1$ vs TEMED concentration is shown for both gel samples. The important features are:

(i) In the absence of TEMED, R is positive, demonstrating a net charge translocation in the forward direction.

(ii) The largest absolute value of R where the sign of R is negative is equal in magnitude with the reverse case when the sign of R is positive (within the 10% experimental error). This is considered as evidence for the complete reversal of the proton pump.

(iii) The concentration dependence of the R values is unusual: the largest negative R is reached at 40–50 μM TEMED for both gel samples, after that a tendency to re-establish the original pumping direction appears. The slope of the rise of R is proportional to the bR concentration.

The simplest interpretation of these results is to assume 2 TEMED-binding sites: the first (site A) is responsible for the reversal and the second (site B) for the re-establishment of the pump direction.

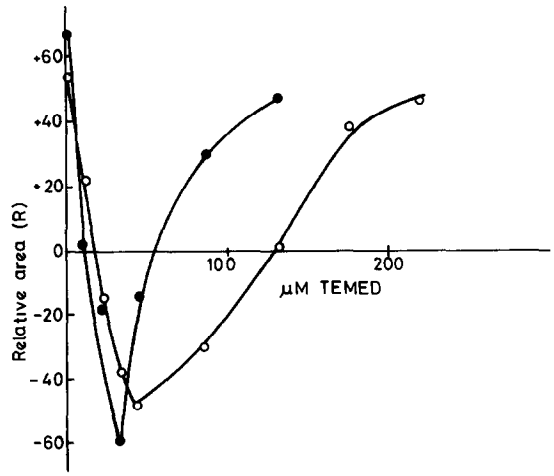

Fig.3. Dependence of the relative area, $R = -\Sigma A_i/A_1$ (where ΣA_i is the area of the different components of the electric signal) on TEMED concentration. bR concentration: (●) 90 μM; (○) 360 μM.

Sites A need further explanations. Because the TEMED concentration at which the reversal occurs is more or less independent of the bR concentration we have to assume that all the A binding sites are occupied by TEMED molecules, i.e. in the case of ~150 ml solution and 1 day of soaking the small piece of gel in it, the binding sites are saturated with TEMED. This explanation was confirmed in another experiment where both the lower and higher bR concentrated gels (90 and 360 μM bR, respectively) were put into ~50 μM TEMED solution for increasing time durations and the build up of the negative R value was recorded. The soaking time needed to reach the negative R value was ~4-times larger in the case of the gel of higher bR concentration than for the gel of lower bR concentration. In the case of the measured points at <50 μM TEMED the 1 day soaking time was not enough for saturating the A sites. (The data also show that TEMED diffuses slowly into the gel.)

(iv) Repeated washing of TEMED-containing samples restores normal behaviour, showing that the molecules do not bind covalently. Furthermore, the TEMED effect may be abolished by the addition of salts. Fig.4 shows the restoration of the normal pump by adding $CaCl_2$ or NaCl to the bathing solution containing 50 μM TEMED.

(v) To show that the reversal of the direction of the proton pump is not due to the presence of the gel, we also measured the effect of TEMED, in oriented pm suspension. The result was similar to those in the gels.

The observations suggest a simple hypothesis to explain the influence of TEMED (and in general the diamines) on the bR proton pump. The diamines are positively charged in solution and therefore are easily adsorbed by the negatively charged groups (A sites) near to the external surface of pm-s [8]. The surface potential is influenced by this adsorption and results in a reversal of the direction of proton flow. It is interesting to observe the 2 time-resolved components (V_F, V_S) (fig.1b) which may represent the motion of protons in the 2 long paths from the Schiff base region to the internal side and from the external side to the Schiff base region. At increasing TEMED concentrations, the adsorption sites (B sites) near to the internal surface become gradually occupied. This balances out the reversing action which the TEMED molecules at the external surface have on the surface potential.

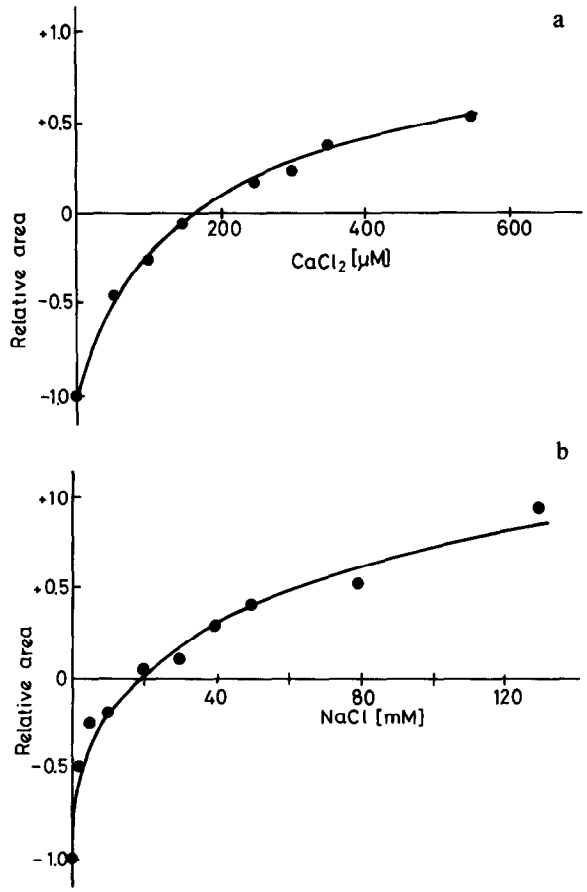

Fig.4. Dependence of the relative area R (see text) on the added concentrations of (a) $CaCl_2$ and (b) NaCl. 90 μM bR and 50 μM TEMED were used for both measurements.

Electrogenesis of bR chemically modified by ethylenediamine was studied by Ovchinnikov et al. [9]. The chemical modification did not reverse the direction of the generated photopotential but did influence the kinetics in pm-s. In reconstituted vesicles, however, the direction of the photovoltage was opposite to that of non-modified bR vesicles. These observations were interpreted as being due to the replacement of negatively charged free carboxyls by positively charged ethylenediamines. Our data and our hypothesis show that the charge states of some groups near to the surface of pm-s (very probably carboxyl groups) can also be modified by the simple, reversible adherence of diamines.

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