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Cooperativity-regulated parallel pathways of the bacteriorhodopsin photocycle

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Abstract The paper demonstrates that the actinic light density dependence of the millisecond part of the bacteriorhodopsin (BR) photocycle at high pH predicts a model, which is the same in the sequence of the intermediates as concluded previously on the basis of double flash experiments [1992, FEBS Lett. 311, 267–270]. This model consists of the $M_f \rightarrow N \rightarrow BR$ and $M_s \rightarrow BR$ parallel pathways, the relative yields of which are regulated by cooperative interaction of the BR molecules. The decay of M_s is always slower than the decay of M_f and described as a direct reprotonation of the Schiff-base from the bulk, and the recovery of the ground-state nearly at the same time. M_s is decomposed into M_f' and M_s'. The first does not reprotonate, and similarly to M_f, it is suggested to be before the conformational change (switch), which latter process would be just before the decay of M_f. A simple way for the determination of the kinetics is also used. This confirms that the amount of N decreases with increasing fraction cycling and shows that the decay rate of N is independent of the fraction cycling. The differences in the kinetics are compared to each other, and they seem to allow a new way of kinetic evaluation at least under special conditions. The aim of this paper was briefly explained in my poster presented on the VIth International Conference on Retinal Proteins (see [14]).

Key words: Kinetic difference; Kinetics of N; Light intensity dependence; Photocycle model; Proton translocation mechanism

1. Introduction

The proton translocation mechanism of the light-driven proton pump bacteriorhodopsin (BR) is not fully understood. Many details are known about which residues participate in the proton translocation mechanism, but the timing and the interconnection of the processes – or simply the reaction kinetic model of the photocycle – is under debate [1].

A recently rediscovered effect, the actinic light density dependence of the photocycle kinetics [2] and the origin of it, a photocooperativity among the neighboring BR molecules in the purple membrane [3], will probably affect the solution of this latter problem: in the case of a sample containing the mixture of the photocycling molecules with and without photocycling neighbors, the appearance of at least two parallel pathways seems to be natural. One pathway corresponds to the unaltered photocycle and the other to the altered one.

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Abbreviations: BR, bacteriorhodopsin; M_f, M_s and N, intermediates of the photocycle; M_f and M_s, rapidly and slowly decaying components of the M intermediate; M_f' and M_s', subforms of the M_s intermediate.

In the present state of the description of intramolecular events of the proton pumping no model has been developed to account for the difference between the altered and the unaltered photocycles (see e.g. [4]). Moreover, no phenomenological model suggested by the actinic light density dependence of the photocycle kinetics [2] has been selected. The results presented here are devoted mainly to the solution of these two questions.

Additionally, the properties of the actinic light density dependence will be shown to suggest a very simple alternative evaluation method based on kinetic differences, that is different from the other, similar attempts [5,6].

2. Materials and methods

The absorption kinetic measuring system and the preparation of the sample were the same as described previously [2]. The exciting light was obtained from a flash lamp pumped dye laser (1 μs half-duration, λ_e = 590 nm). An exception is that in order to analyze the model predictions of the previously characterized actinic light density dependencies [2] for Figs. 1 and 2 the data were taken for processing from that study. Those measurements applied a 10 ns long flash at 505 nm from an excimer laser pumped dye laser.

3. Results and discussion

3.1. Pathways in the millisecond domain of the BR photocycle at high pH

In one of our recent studies we determined the kinetics of the N intermediate and the recovery of the BR ground-state [7]. The double excitation method used there cannot be applied conveniently to study the influence of the actinic light density on the photocycle. At low light intensity the signal to noise ratio would be too small.

The determination of the kinetics of the BR recovery is possible in an alternative way because at 330 nm the kinetics coincide with the kinetics of the BR recovery [8], since the 330 nm absorption change is due to the 13-*cis* chromophore (see e.g. [4]) of the photocycle intermediates.

The absorption changes accompanying the photocycle at 335 and 412 nm are shown in Fig. 1. at different actinic light densities. This figure shows that parallel to the increase in the amplitude of the second phase of the M decay (measured at 412 nm) the recovery of the BR ground-state becomes faster.

In one of our previous studies [2] we have shown that these kinetics can be described in the millisecond time domain by 3 components designated as M_f, M_s and N. The kinetics at 412 nm consist of M_f and M_s, while the kinetics at 335 nm consist of M_s and N. The relative weights of these components are influenced by the strength of the optical excitation, but the lifetimes of the components remain constant [2].

In Fig. 2 the relative weights of M_s are shown at the two above mentioned wavelengths as the function of the actinic

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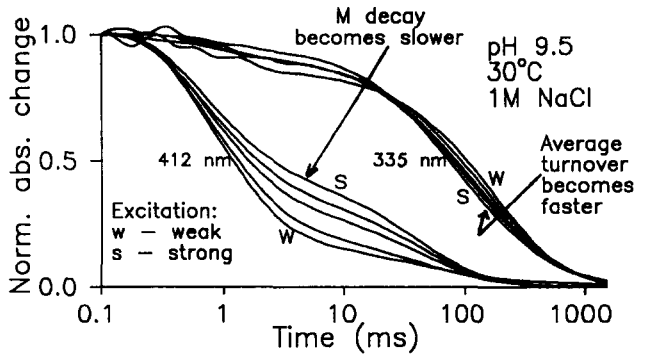

Fig. 1. The normalized absorption changes at 335 and 412 nm. (The small differences in the 0.1 to 20 ms time domain of the 335 nm traces are due to only a few percent contribution of M_f to the absorption changes at that wavelength.)

light density. The correlation of the relative weights of M_s is very good at any actinic light density. Thus this figure suggests that those molecules which disappear with the slow component of M immediately appear as ground-state BR. This result predicts that the M_s component is the decay of an intermediate (the decay of the M_s intermediate) to the ground-state.

The remaining two components, M_f and N, of course have the same light intensity dependencies which is the opposite of the one for M_s. Taking into account that M_f does not lead to the recovery of the ground-state (335 nm), the molecules decaying by M_f can get into the ground-state only by the N decay. As N practically does not contribute to the M kinetics (412 nm), the corresponding two subsequent intermediates cannot be in significant equilibrium.

Briefly the actinic light density dependence of the photocycle kinetics at high pH predicts a model consisting of $M_f \rightarrow N \rightarrow BR$ and $M_s \rightarrow BR$ parallel pathways. This arrangement of the intermediates has been suggested previously on the basis of double excitation experiments [7].

The actinic light density dependence of the photocycle kinetics originates in a cooperative interaction of the neighboring BR molecules [3], thus the yields of the parallel pathways are regulated by the cooperativity.

Additionally, it is worthy to note that it would be difficult to explain the above mentioned observations even by the latest version of the equilibrium models of the high pH BR photocycle. Although that model [11] is better in the description of the kinetic data compared to its preceding form, the suggested single $M_2 \leftrightarrow N^{(-1)} \rightarrow N^{(0)} \rightarrow BR$ pathway predicts a BR recovery different from the observed one: If we accept that $N^{(-1)}$ and $N^{(0)}$ are spectrally indistinguishable, the decay of $N^{(-1)}$ to $N^{(0)}$ should correspond to a component with a negative amplitude factor in contrast to the observation that both of the two BR recovery components have positive amplitudes (see also [7]).

3.2. Some features of M_f and M_s

Fig. 3 shows the otherwise well-known fact that at high pH the lifetime of M_s is close to proportional to the reciprocal of the proton concentration, while at lower pH values this dependence practically disappears [9, 10]. As a result of this the pH dependence of the lifetime of M_s at high pH seems to indicate that the decay of M_s is a proton uptake process from the bulk, and in this respect it is similar to the decay of N. On the other hand, it is obvious that at lower pH the decay of M_s may not be limited by this proton uptake process.

One of the basic features of the model suggested in the present study for the millisecond part of the high pH BR photocycle is that the lifetimes of the components are independent of the fraction cycling. In the M kinetics this means that M_f is gradually replaced by M_s with increasing fraction cycling. This is shown in Fig. 4 by that the difference between the normalized pairs of the M kinetics for weak and for strong excitations have the same shape.

For high pH on the basis of the previously published data [2] the coincidence of these differences is not surprising (data not shown). The interesting result is that this is true also at pH 5, where the lifetimes of the components differ from each other only by a factor of less than 3 (see Fig. 3). This indicates that the actinic light density dependence of the M kinetics is not due to a local pH change, and it is probably not due to a change in the extent of a backreaction, as in these latter cases due to the changes in the lifetimes (necessary for the description of the alteration of the kinetics by light) a shift in the rise or decay of the differences would be expected. On the contrary, if the photocycle passes through a different pathway when the neighbor of the photocycling molecule is also photocycling, this is able to explain the findings.

It is important that the decay of M_s remains slower than the decay of M_f at this low pH value as well, even though at high pH the rate of the slow M decay is (as known from [9,10]) proportional to the proton concentration. This feature can be used in the physical interpretation of the $M_s \rightarrow BR$ pathway.

3.3. Cooperativity-regulated proton translocation mechanism of BR

The generally accepted point of view with respect to the steps of the proton pumping is as follows: during the series of the conformational changes of the functioning BR molecule the proton is transferred from the Schiff-base to a proton acceptor (Asp^{85}) during the formation of the M intermediate. Then a proton is taken up from the proton donor (Asp^{96}) by the Schiff-base (decay of M, and rise of N), and in the final step of the photocycle the proton donor takes up a proton from the bulk (decay of N) [4]. Note, that at acidic and neutral pH the photocycle and the proton transfer mechanism is complicated by the

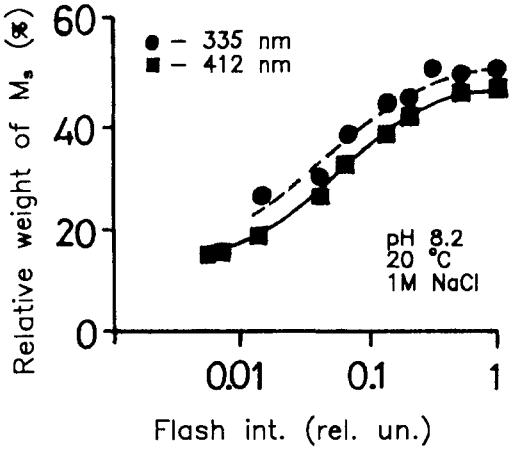

Fig. 2. The contribution of M_s to the M decay and to the BR recovery.

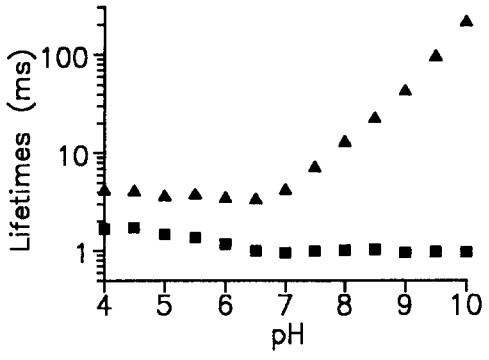

Fig. 3. The lifetimes of the fast and slow components of the M decay.

presence of the O intermediate (and sometimes more than one N intermediates are supposed [11]).

In the model defined in the present study, the above discussed mechanism describes only the $M_f \rightarrow N \rightarrow BR$ pathway. Another way of the reprotonation of the Schiff-base can be defined by assuming that the proton can also be taken up directly from the bulk. (Such process appears in the case of those mutants in which the proton donor (Asp^{96}) has been removed [9].) This direct reprotonation corresponds to the $M_s \rightarrow BR$ pathway. The pH dependence of M_s (Fig. 3) supports this suggestion. The fact, that the proton donor (Asp^{96}) may not function in this case, predicts, that the cooperative interaction inhibits the proton release by the proton donor (Asp^{96}). Note that according to [3] this inhibition should originate in a conformational change induced by one specific neighboring photocycling BR molecule.

On the basis of that the decay of M_s is never faster than the decay of M_f it seems to be probable that a process related to the decay of M_f should be involved also in the pathway of M_s, although this process remains spectrally invisible. This can be a large conformational change which allows the fast jump of the proton from the donor to the Schiff-base in the case of M_f, and it allows the proton uptake from the bulk in the case of M_s.

As a consequence, the $M_s \rightarrow BR$ pathway can be decomposed into $M'_f \rightarrow M'_s \rightarrow BR$, where according to the experiments M'_f is spectroscopically (and possibly kinetically) indistinguishable from M_f, and M'_s corresponds to such a state in which the large conformational change has appeared but the Schiff-base is still deprotonated (the Schiff-base is like in M, but the protein part is like in N). Such state of BR has been observed in a recent study [12].

One of the interesting questions is why M_s is well detectably slower than M_f at acidic pH, where M'_s should be very fast. There are two possible explanations. One is that M'_f is slower than M_f, i.e. the BR molecule which is perturbed by its neighbor is slower in changing its conformation. The other explanation is if the diffusion of the proton to the Schiff-base through the protein channel is responsible for this time delay. (Since I do not believe that the proton conducting channel would be open before the decay of M_f, I consider the latter reason more feasible.)

3.4. The kinetics of the N intermediate

The predictions of the model consisting of the fraction-cycling-regulated $M_f \rightarrow N \rightarrow BR$ and $M_s \rightarrow BR$ parallel pathways can be confirmed in some further details without referring to exponential components. Some details of this are shown bellow.

According to the current point of view, in the millisecond part of the photocycle of BR at high pH, besides M and the ground-state, only the N intermediate is present in a considerable amount [4]. The kinetics of M can be measured at 412 nm, and when the amount of M is maximal, practically no other intermediate is assumed to be present in the sample. At other wavelengths, where N also contributes to the signal, the absorption changes are altered compared to 412 nm. This change in the shape of the absorption changes exhibit the kinetics of N. Thus the calculation of the difference between the normalized kinetics provides a very simple way for the determination of the N kinetics.

These differences are calculated from the 412 and 570 nm data, and shown in Fig. 5a for different actinic light densities. This figure indicates that the amount of N decreases with increasing actinic light density. This is in accordance with the results of Drachev and co-workers [13], and Fig. 5a is a more convincing evidence for this, than obtained from Fig. 4 of [2],

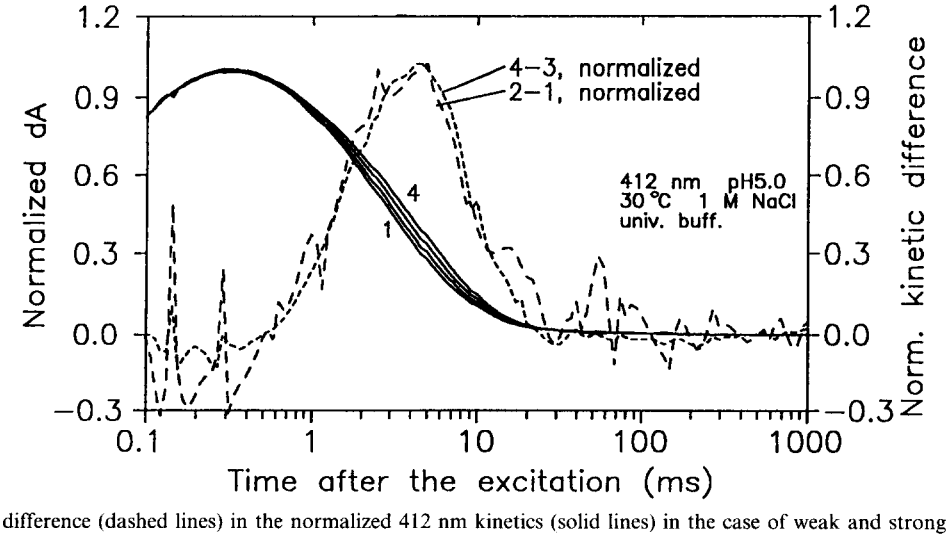

Fig. 4. The difference (dashed lines) in the normalized 412 nm kinetics (solid lines) in the case of weak and strong excitation.

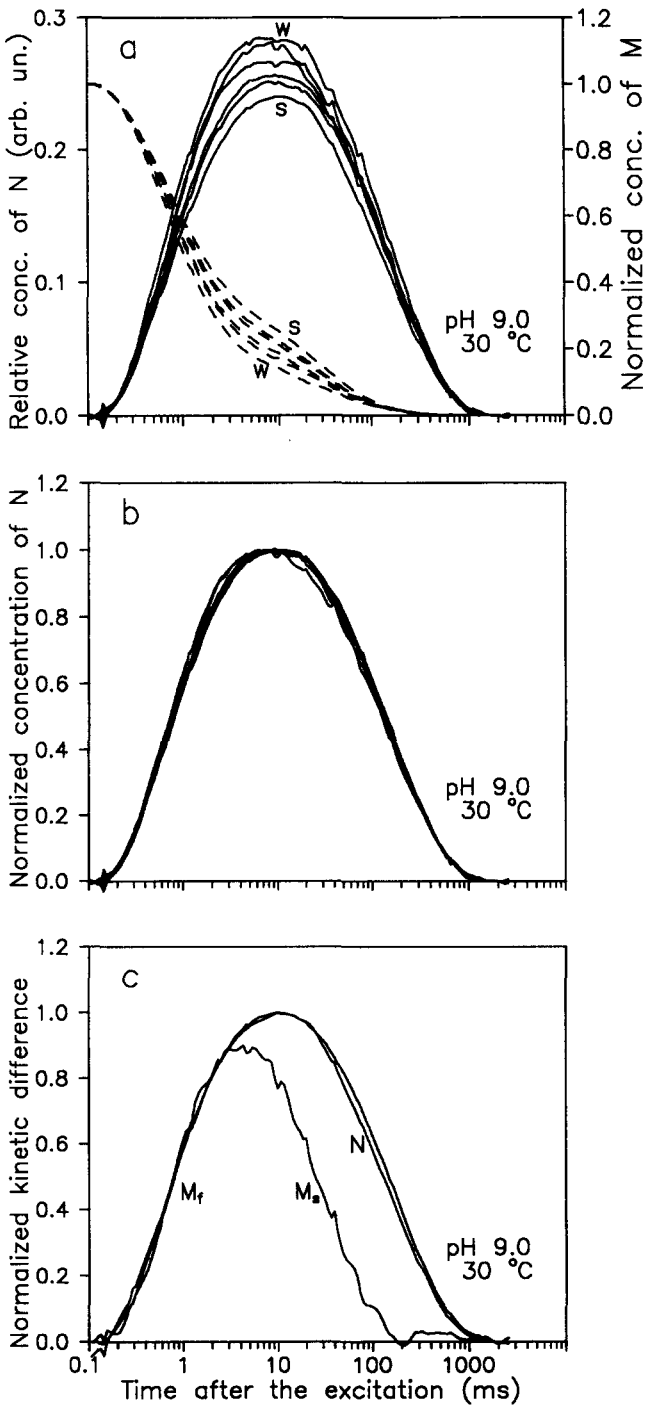

Fig. 5. The kinetics of N calculated from the differences in the normalized kinetics (a) before and (b) after normalization. (The dashed lines show the corresponding M kinetics.) (c) The kinetics by which M_f can be exchanged to N, and M_f to M_s. The first one is calculated from the difference in the normalized kinetics measured at 570 and 412 nm, and similarly from the 330 and 412 nm data. The second one is calculated similarly to Fig. 4.

where exponential components were fitted to similar absorption kinetic data.

Fig. 5a allows a quantitative comparison of the extent of the change in the M and N kinetics. For this an appropriate time is when the conversion of M to N is almost maximal, but the recovery of the ground-state is still negligible. In the present case 4 ms was chosen. There the change in the proportion of M decayed and N accumulated correspond to each other. They show 20 and 22% increase, respectively, if the excitation is decreased from the strongest to the weakest one. By this it is shown that the fastly decaying part of the M intermediate and the amount of N formed are proportional to each other.

The kinetics of N shown in Fig. 5a can be normalized, in order to check whether or not the kinetics of N depend on the actinic light density. Fig. 5b shows that these kinetics are identical within the experimental uncertainty. Thus the lifetime of N is independent of the fraction cycling. Note that this result is obtained here without fitting of exponentials, and in this respect it is similar to the ones shown in Fig. 4.

3.5. *Differences in the normalized kinetics*

The two different types of the differences in the normalized kinetics derived above can be compared to each other for the demonstration of the different processes in the millisecond time domain of the high pH BR photocycle.

The increase in the actinic light density exchanges a certain part of M_f to M_s in the normalized absorption changes (see Fig. 4). The difference in these curves is a kinetics rising with the M_f and decaying with the M_s components. Thus by the addition of this kinetics to the absorption changes measured under the same circumstances but at any arbitrary wavelength, one can exchange some M_f to M_s, and vice versa.

Similarly the kinetics of the N intermediate is expected to rise with M_f and to decay with the N component. This difference is appropriate for the exchange of M_f to N, and vice versa.

These kinetics can be compared, and they are shown in Fig. 5c. After taking into account that the peak of the M_f–M_s kinetics is smaller due to kinetic reason, one can see that the rise of the two kinetic differences are the same. Their decay, of course, is different, and a simple readout it can result the lifetimes of M_s and N. (In the present case these are 1, 40, and 200 ms for M_f, M_s and N, respectively.)

Note that these differences could be used for the evaluation of absorption changes. For example both M_s and N could be transformed into M_f, and the amplitudes of the kinetic differences necessary for this transformation would reflect the amplitudes of M_s and N. (Note that Fig. 5c also shows that it is indifferent whether we use the 330 or the 570 nm (or several other wavelengths) absorption kinetic data for the calculation of the N kinetics.)

Similar consideration to the above mentioned ones may provide a process oriented way of the evaluation instead of the exponential component oriented one.

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