ISOTOPE EFFECTS AND ACTIVATION PARAMETERS FOR CHEMICALLY MODIFIED BACTERIORHODOPSIN

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1. Introduction

As a means towards determining the mechanism of proton translocation in bacteriorhodopsin (BR), we have been investigating the effects of chemical modification of specific amino acid residues on the kinetics of the BR photocycle [1–5]. Some of the modifications studied include tyrosines modified by iodination [1,2], tryptophans by reaction with *N*-bromosuccinimide (NBS) [3], lysines by reaction with dimethyl adipimidate (DMA) [4] and ethyl acetimidate (EA) [5], arginines by treatment with 2,3-butanedione (BD) and phenylglyoxal (PG) [6], and carboxyls with 1- ethyl-3- (4 azonia- 4,4- dimethylpentyl) carbodiimide iodide (EAC) [6].

Suspension of normal purple membrane of *Halobacterium halobium* in D_2O in the light results in the rapid exchange of 40% of the protons in the BR molecule, in particular the proton attached to the Schiff-base nitrogen [7]. The effect of this deuterium exchange on the rise and decay of the M_{412} photocycle intermediate has been reported [8–10].

Here, studies of isotope effects on chemically modified BR are presented and from the temperature dependence of the rate constants the activation parameters for the rise and decay of the M_{412} intermediate have been evaluated in each case. The results show that chemical modifications do not substantially change the isotope ratios for the rise (3.4–5.5) and decay (1.4–3.5) of M_{412} indicating that the basic mechanism for the formation and decay of M_{412} is unaltered by chemical modification. The activation parameters for the rise of M_{412} are less affected by chemical modification than are those for the decay process, which are changed markedly on modification with BD, PGO, I_2 and EAC. These modifications must change the BR molecule in such a way that reprotonation of the Schiff-base requires considerable molecular reorganization. In addition, measurements of the M_{412} species in the photostationary state are qualitatively correlated with the decay rate of M_{412}.

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2. Materials and methods

Purple membranes were chemically modified using the methods in [1–6]. Fully deuterated BR purple membranes were a kind gift from Dr H. Crespi. Suspensions were made at 0.15–0.30 mg protein/ml in H_2O or D_2O after a minimum of 3 washes by centrifugation at 210 000 × *g* for 15 min at 5°C followed by resuspension in 3 ml D_2O or H_2O. The final solutions were 100 mM in KCl and were buffered to pH 7.5–8.0 with 5 mM phosphate buffer. For liposome experiments, BR was reconstituted into partially purified azolectin at a 1:20 protein/lipid (w/w) ratio by sonication in a bath-type sonicator. M_{412} in the photostationary state was measured in an Aminco DW-2 spectrophotometer by side actinic illumination through a Corning 3- 67 low wavelength cut-off filter; the photomultiplier was protected by a Baird Atomic 412 nm transmission interference filter. The formation and decay of M_{412} was measured using a flash photolysis apparatus; flashes were generated with a phase-R dye laser with rhodamine 575 (0.2 J/flash, 150 ns flash risetime). The rise and decay of M_{412} was collected with a Biomation 1010 transient recorder interfaced to a PDP 11/34 computer, using a monochromatic measuring beam. At each temperature, the rise and decay of M_{412} was averaged from a

minimum of 10 flashes repeated at least twice. The data was analyzed via the computer assuming that the rise kinetics were due to a single first-order process to give a rate constant k_1. The decay kinetics were often found to consist of more than one first-order process; in such cases, a curve peeling technique was used to analyze the data in terms of rate constants k_2 and k_2. Activation parameters were obtained from plots of ln$k$ versus $1/T$.

3. Results and discussion

3.1. *Isotope ratios*

The half-lives for the rise and decay of the M_{412} intermediate for suspensions of control and chemically modified samples in both H_2O and D_2O are shown in table 1. In essential agreement with studies [8–10] on unmodified BR, the rise and decay kinetics have isotope ratios of 4.8 and 2.1, respectively. Almost identical ratios are also found for fully deuterated BR (gift of H. Crespi) in D_2O and H_2O. Additionally, we have found that BR reconstituted into asolectin liposomes shows changed photocycle kinetics with a decreased rise and increased decay time. However, the isotope effects remain similar to those of the purple membrane patches. The D_2O effects on the photocycle are fully consistent with the hypothesis correlating the rise and decay of M_{412} to the deprotonation and reprotonation of the Schiff-base which links the retinal chromophore to the BR molecule. As can be seen from table 1, the rise kinetics are only slightly varied with chemical modification, whereas the decay kinetics may change by up to two orders of magnitude. In both cases, isotope effects were still observed, indicating that the basic mechanism remains similar in the modified samples. It should be noted that the decay kinetics of many of the samples cannot be analyzed in terms of a simple monophasic first-order decay; at least two first-order processes are present in many cases. Possible explanations for this observation include heterogeneity of the chemical modification or more than one pathway for M_{412} reprotonation.

3.2. *Activation parameters*

Temperature dependence studies of the rates of rise and decay of M_{412} enable one to calculate the activation parameters for these processes. Our studies were done over 5–50°C. The results in table 2 are the average of samples in both H_2O and D_2O since only small differences occur between them. Under the conditions of our experiment, breaks in the Arrhenius plots at ~30°C were only observed in control, in NBS- and EA-modified and possibly in the DMA-modified samples. All the other samples showed linear plots over the whole temperature range. The breaks which we observed for control samples were not as pronounced as those in [8,9], possibly because of different sample conditions (pH, buffer). These breaks have been interpreted [9,11] in terms of either a solid–liquid phase transition of lipids which, however, are not detected by differential scanning calorimetry [12,13] or by a change in conformational state of the protein. Those samples which do not show a break in the Arrhenius plot in our experiments are those which have the greatest effect on the photocycle or in which the lipid environment is changed (liposome sample) and are consistent with either of the above hypotheses.

Table 1
Kinetic parameters and isotope ratios for rise and decay of M_{412}

Bacteriorhodopsin sample	Rise[a]			Decay (slow phase)			Decay (fast phase)		
	$t^H_{1/2}$	$t^D_{1/2}$	k_1^H/k_1^D	$t^H_{1/2}$	$t^D_{1/2}$	k_2^H/k_2^D	$t'^H_{1/2}$	$t'^D_{1/2}$	k'^H_2/k'^D_2
Unmodified	0.069	0.33	4.8	3.65	7.70	2.1	–	–	–
Unmodified (liposomes)	0.016	0.051	3.2	47.8	110	2.3	–	–	–
EA (lysine)	0.067	0.30	4.5	5.54	15.4	2.8	–	–	–
NBS (tryptophan)	0.043	0.147	3.4	19.2	67.3	3.5	3.5	9.2	2.7
DMA (lysine)	0.072	0.35	4.9	33.0	46.2	1.4	5.9	–	–
EAC (carboxyl)	0.098	0.42	4.2	90	131	1.5	12.0	23.0	1.9
I_2 (tyrosine)	0.026	0.11	4.2	420	1042	2.5	64	118	1.9
BD (arginine)	0.112	0.62	5.5	384	1170	3.0	71	221	3.1
PGO (arginine)	0.133	0.71	5.4	308	724	2.3	53	145	2.7

[a] Half-lives in ms; temp. 20°C

Table 2
Activation parameters for rise and decay of M_{412}

Bacteriorhodopsin sample	Rise[a]			Decay		
	E_a	$S^{\ddagger}$	$\Delta G^{\ddagger}_{293}{}^{\circ}$	E_a	$S^{\ddagger}$	$\Delta G^{\ddagger}_{293}{}^{\circ}$
Unmodified	17.7	3.0	16.8	18.3	−2.0	18.8
Unmodified (liposomes)	12.8	−10.4	15.8	14.7	−18.9	20.1
EA (lysine)	18.3	5.0	16.8	22.5	11.2	19.2
NBS (tryptophan)	16.6	1.0	16.5	19.2	−2.0	19.5
DMA (lysine)	15.5	−3.0	16.4	18.5	−5.2	19.9
EAC (carboxyl)	15.8	−4.0	17.0	11.4	−31.7	20.6
I_2 (tyrosine)	15.0	−5.6	16.6	10.5	−38.0	21.7
BD (arginine)	16.9	−0.8	17.2	10.6	−38.0	21.7
PGO (arginine)	15.0	−7.8	17.2	10.7	−37.0	21.5

[a] Results are averages of samples in H_2O and D_2O. E_a and $\Delta G^{\ddagger}_{293}{}^{\circ}$ in kcal/mol, error ± 1 kcal/mole, $S^{\ddagger}$ in *E*.u/mol, error ± 5 *E*.u/mol

The general results which emerge from the activation parameters are that in all cases the free energy of activation for the rise is several kcal/mol less than that for the decay. The parameters for the rise of M_{412} are less affected by chemical modification than those of the decay. The small differences observed for the rise of M_{412} are difficult to interpret physically and it therefore appears that the chemical modifications studied to date have only minor effects on the deprotonation of the Schiff-base. The effects on the decay of M_{412}, associated with the reprotonation of the Schiff-base, are much more sensitive to chemical modification. It is particularly striking that those modifications which lengthen the decay of M_{412} most significantly, i.e., BD, PGO, I_2, EAC, also show the greatest effect on the activation parameters. This is most apparent in the change of the entropy of activation from −0.2 E u/mol in control to −35.0 E u/mol for those samples. This shows that these particular modifications change the BR molecule in such a way that the reprotonation of the Schiff-base requires considerable molecular reorganization. Specifically, the I_2 modification iodinates tyrosines *ortho* to the hydroxyl group [14]. If the tyrosine residue is directly involved in the reprotonation of the Schiff-base, the effect of this modification on the acti-

Table 3
Photostationary steady state of M_{412} compared to decay kinetics of M_{412}

Bacteriorhodopsin samples	Mole ratio of 412/570 cf. control	$t_{1/2}$ (sample) / $t_{1/2}$ (control)	412/570 ratio D_2O/H_2O	$t^{D}_{1/2}/t^{H}_{1/2}$
Unmodified	1.0[a]	1.0[b]	3.2[c]	2.1[d]
Unmodified (liposomes)	12.9	13.1	–	–
EA (lysine)	2.5	2.5	2.5	2.8
NBS (tryptophan)	9.2	5.3	1.7	3.5
DMA (lysine)	9.6	9.0	2.4	1.4
EAC (carboxyl)	10.4	24.6	1.2	1.5
I_2 (tyrosine)	70.9	115	3.9	3.0
BD (arginine)	26.0	105	2.2	2.3
PGO (arginine)	34.0	84	1.6	2.5

[a] Obtained from absorbance measured at 570 nm where $\epsilon_{570} = 63\,000\ M^{-1} \cdot cm^{-1}$ [15] and at 412 nm where $\Delta\epsilon_{412} = 32\,800\ M^{-1} \cdot cm^{-1}$ [16], values given are for H_2O solutions

[b] From slow phase decay data in table 1

[c] Ratio of 412/570 ratios calculated in D_2O and H_2O

[d] Values obtained from table 1

vation parameters is understandable in terms of a change in the pK of the hydroxyl proton which lowers E_a and an increase in steric effects leading to a decreased $S^{\mp}$.

The BD and PGO modifications both alter arginine residues which are believed to be involved either in the formation of salt bridges which maintain the conformation of BR or in the proton pathway through the BR molecule [6]. Modification of arginine could thus bring about either significant conformational changes or change the proton pathway, both of which could lead to the observed negative $S^{\mp}$ for the reprotonation step. However, other evidence [5] suggests that the conformation of BD-modified BR is little changed from control and hence the major effect may be on the proton pathway.

EAC modifies carboxyl groups but is also believed to lead to intramolecular crosslinking between lysines and carboxyls. Such crosslinking could again result in significant structural changes in the BR molecule or in changes in the proton pathway. Since DMA, which is known to crosslink, does not affect the activation parameters as much as EAC, perhaps the change in the proton pathway is again the more important process.

3.3. M_{412} *photostationary state*

The photostationary steady state concentration of M_{412} is larger in D_2O than in H_2O for all the samples studied by a factor of 1.2–3.9 (see table 3). This observation is most simply explained in terms of an unbranched photocycle in which only the formation and decay of M_{412} are significantly isotope-dependent and if one also assumes that only a small fraction of BR is actually photocycling. Under these conditions, one would predict that the steady-state concentration of M_{412} under constant illumination should be directly proportional to the half-time of the decay process. As can be seen in table 3, the agreement of this prediction with the chemically modified samples is at least qualitatively correct. Additionally, the M_{412} steady state concentration ratio in D_2O/H_2O should be proportional to the ratio of the decay half-lives in D_2O and H_2O ($t^D_{1/2}/t^H_{1/2}$). This agreement, as shown in table 3, is also qualitatively observed.

A further extension of this hypothesis suggests that since the M_{412} concentration is predicted to be inversely proportional to the decay rate, that the M_{412} steady state concentration should decrease with increasing temperature and that an Arrhenius plot of the M_{412} steady state absorbance against $1/T$ should give an activation energy similar to that obtained from the temperature dependence of k_2. For unmodified samples in both H_2O and D_2O, the M_{412} concentration does indeed decrease with temperature and from the slopes of the Arrhenius plots activation energies of 16.5 ± 1 and 17.0 ± 1 kcal/mol are calculated in the excellent agreement with the values of 18.1 ± 1.0 and 18.7 ± 1.0 obtained from the k_2 data.

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