# Prolonged lifetime of the M intermediate in D96N-mutant bacteriorhodopsin films enhanced by diaza-15-crown-5

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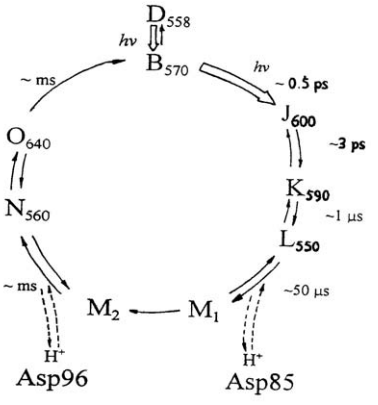
In order to get even longer M intermediate lifetime for the purpose of expanding the use of bacteriorhodopsin (BR) materials in optical applications, the combination of genetic engineering and the use of chemical additives was attempted. Different compositions of BR (wild type and D96N mutant)–PVA films with diaza-15-crown-5 (1,4,10-trioxa-7,13-diazacyclopentadecane) additive were prepared. Spectral and kinetic measurements were carried out at room temperature. It was observed that the additive contents that could be used in these films were limited. The highest BR : diaza-15-crown-5 molecular ratios were 1 : 250 and 1 : 150 for $BR_{WT}$ and $BR_{D96N}$ films, respectively. Further additions resulted in permanent damage to the purple membrane and a loss of photochromism. The kinetic curves of the M state decay for each film were fit, in a least-squares fashion, by a three-exponential function to obtain sufficiently small residuals. The fitting results indicate that for both types of BR molecules, the decay of the M state was slowed down gradually with increasing additive content. At a $BR_{D96N}$ : diaza-15-crown-5 molecular ratio of 1 : 150, the BR film had the longest lifetime and its photochromism could be observed for about one and a half hours. This indicates that the combination of genetic engineering and chemical additives is more efficient than using either of these two methods. The main reason for this significant prolongation may originate from the strong basic and $H^+$ complexing properties of diaza-15-crown-5 and the change of the membrane surface charge caused by this additive.

Bacteriorhodopsin (BR) is the sole protein component of the purple membrane (PM) of *Halobacterium salinarum*[1] and it functions as a light-driven proton pump.[2] BR is composed of seven transmembrane α-helices enclosing the binding pocket for an *all-trans* retinal chromophore, which is bound to Lys-216 *via* a protonated Schiff base.[3] Upon absorption of light, the retinal isomerizes from the *all-trans* to the *13-cis* conformation.[4] This triggers structural change,[5] in the BR molecule and protons are pumped from the cytoplasm through the halobacterial cell membrane to the outer medium, thus generating[6] a transmembrane proton gradient.[6] The structure and function of this membrane protein have been intensively investigated over the last three decades. Reviews summarizing the current knowledge on the biochemical and photophysical properties of BR have been published.[7]

For every proton pumped out of the cell, the BR molecule undergoes a photocycle (Scheme 1) that contains a series of intermediate states with different absorption spectra and various lifetimes.[8] Of all these photointermediates, the M state is the only one that has a deprotonated Schiff base, and its absorption spectrum is blue-shifted about 160 nm from the initial B state. Aspartic acid 85 (Asp-85) is involved in this deprotonation of the Schiff base. During the reprotonation of the Schiff base, which occurs in the decay of the M state, Asp-96 serves as a proton donor. After the reprotonation of Asp-96 from the outer medium, the retinal chromophore undergoes a configurational change and the B state is finally reached, which differs from the M state not only in the configuration but also the protonation state of its chromophore.[9]

Based on this photocycle, the photochromism of $B_{570} \leftrightarrow M_{412}$ ($B_{570} \leftrightarrow M_{412}$ cycles of 1 million times has been reported[10]) is quite unique and provides a mechanism for optical applications.[11] However, the M state lifetime is only on the order of 10 ms in aqueous native BR solutions. Some applications require information storage times significantly longer than seconds to obtain higher light sensitivity, higher contrast ratios, and higher contrast decay times.[12] Hence, how to increase the M state lifetime to minutes under ambient conditions continues to be a focus of investigations of BR materials.




**Scheme 1** Scheme of the photochemical and thermal conversions of bacteriorhodopsin. The photointermediates are abbreviated by single letters. Index numbers indicate the absorption maxima of the intermediates.

Over the past three decades, a number of methods have been developed to extend the lifetime of the M state, such as replacing the retinal chromophore by a synthetic analogue,[13] modifying the structure of the molecule itself by genetic variants,[14]

or changing the environment of BR (humidity,[15] pH,[16] temperature,[17] chemical additives,[18] *etc.*). $BR_{D96N}$ is one example of a mutant using conventional mutagenesis or genetic engineering to modify the properties of BR; the longer lifetime of the M state observed is due to the substitution of asparagines 96 (Asn-96) for Asp-96. Another useful method is to use chemical additives. In our previous work, we had used crown ethers as chemical additives to prolong the M state lifetime of $BR_{WT}$, with satisfactory results.[19]

In this paper, for the purpose of pursuing even longer M state lifetimes to expand the use of BR material in optical applications, the combination of the two methods—genetic engineering and chemical additive—was attempted. Here the effect of diaza-15-crown-5 (1,4,10-trioxa-7,13-diazacyclopentadecane, whose structure is shown in Fig. 1) on the prolongation of the M state lifetime of $BR_{WT}$ and $BR_{D96N}$ was studied and compared. The possible mechanism underlying the prolongation is also discussed.

## Experimental

Wild type (WT) PM was isolated from *H. Salinarum* with the preciously described procedures.[20] The powder of the D96N-mutant of BR was a kind gift from Prof. D. Oesterhelt (Max-Planck-Institute of Biochemistry, Martinsried, Germany) and Prof. N. Hampp (University of Marburg, Marburg, Germany). Both $BR_{WT}$ and $BR_{D96N}$ were suspended in distilled water at a concentration of 10 mg $ml^{-1}$ for samples preparation. Then the suspensions were both normally sonicated to get smaller size patches (< 200 nm) to reduce light scattering. Diaza-15-crown-5 was purchased from Aldrich and PVA (polyvinyl alcohol) from Alfa Aesar. The average molecular weight of PVA was 22,000–26,000. The chemicals were of analytical grade and were used directly without further purification.

We prepared different $BR_{D96N}$ suspensions and BR (WT and D96N mutant)–PVA films with and without the diaza-15-crown-5 chemical additive. The pH of the $BR_{D96N}$ suspension was about 6.7. When diaza-15-crown-5 was added with a $BR_{D96N}$ : diaza-15-crown-5 molecular ratio of 1 : 50, the pH reached about 7.5. With increasing additive concentration, the pH of the suspension mixture gradually increased up to 8.0 for a $BR_{D96N}$ : diaza-15-crown-5 molecular ratio of 1 : 150. For the $BR_{D96N}$ suspension without the additive, an NaOH solution (0.01 M) was used to increase the pH of the suspension to 8.0. The BR–PVA films were fabricated as follows: PVA was dissolved by boiling it in a solution of 50 mM Tris [tris(hydroxymethyl)aminomethane] with a concentration of 15% (wt $v^{-1}$). The BR suspension was combined in a 1 : 1 volume ratio with the PVA–tris solution. Then the BR–PVA mixture was spun at 5,000 rpm for 15 min to remove residual bubbles. Films were prepared separately by dropping BR + PVA or BR + PVA + diaza-15-crown-5 on clean quartz substrates, which were placed on a leveled plate at room temperature. The molecular ratio of BR : diaza-15-crown-5 was in a range of 1 : 50–1 : 300. The pH value of the solution was about 8.0. Then films were dried in air for more than 24 h under ambiant conditions. Because of the low water content of dried BR films, it is not feasible to give a pH value for them. Typically, dried films had an optical density (OD) between 0.4 and 0.6.

Spectral and kinetic measurements were carried out on a Javco V-530 UV/Vis Spectrophotometer under ambiant conditions. The excitation light source was a 300 W xenon lamp equipped with a glass optical filter ($\lambda$ = 560 nm). The maximum power density of the excitation light was approximately 5 mW $cm^{-2}$. All samples were previously light adapted for 10 min, and then they were exposed to 560 nm light for 30 s to reach a high M state concentration.

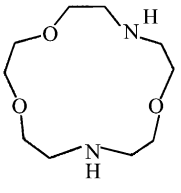



**Fig. 1** Structure of diaza-15-crown-5 (1,4,10-trioxa-7,13-diazacyclopentadecane).

## Results and discussion

### UV spectra of the BR–PVA films

Fig. 2 shows the chemical-additive-induced spectral changes in diaza-15-crown-5 modified $BR_{D96N}$–PVA films. The absorption maximum of the film without diaza-15-crown-5 (shown in Fig. 2, curve b) is located at 565 nm. Compared with the spectrum of a $BR_{D96N}$ suspension (Fig. 2, curve a), the absorption peak is blue-shifted about 5 nm. This blue shift is due to the influence of the dehydration on the Schiff base of the retinal chromophore of BR molecules in a dry film.[21] When diaza-15-crown-5 was added into the $BR_{D96N}$–PVA films, the absorption maximum of the $BR_{D96N}$–PVA films blue-shifted gradually from 564 to 562 nm with increasing diaza-15-crown-5 content over the $BR_{D96N}$ : diaza-15-crown-5 molecular ratio range of 1 : 50–1 : 150 (shown in Fig. 3). For comparison, in a $BR_{D96N}$ suspension with diaza-15-crown-5, the spectral maxima were all located at 570 nm in such an additive concentration range. Since the profile of the spectrum (Fig. 2, curve c) of the $BR_{D96N}$–PVA film with a 1 : 150 ratio is the same as that of $BR_{D96N}$ in suspension, it can be concluded that the physiological activity of $BR_{D96N}$ in such chemically enhanced films is preserved. The slight 3 nm blue shift that is

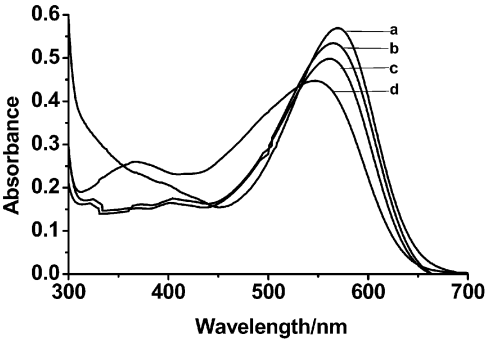



**Fig. 2** Absorption spectra of (a) $BR_{D96N}$ suspension, (b) $BR_{D96N}$–PVA film, (c) $BR_{D96N}$–PVA film with a $BR_{D96N}$ : diaza-15-crown-5 molecular ratio of 1 : 150 and (d) $BR_{D96N}$–PVA film with $BR_{D96N}$ : diaza-15-crown-5 molecular ratio of 1 : 200.

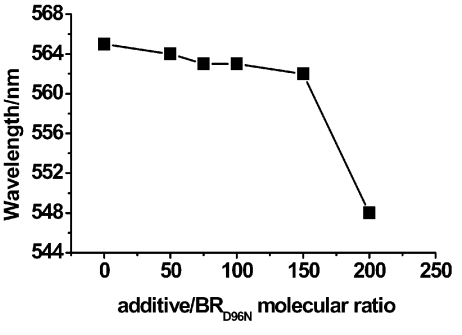



**Fig. 3** The absorption maximum of $BR_{D96N}$–PVA film with different diaza-15-crown-5 concentrations.

caused by adding diaza-15-crown-5 suggests that diaza-15-crown-5 has only a weak interaction with $BR_{D96N}$ in such dry films. In addition, the additive concentrations in these films were limited. In $BR_{D96N}$–PVA films, when the molecular ratio of $BR_{D96N}$ : diaza-15-crown-5 reached 1 : 200, $BR_{D96N}$ was partially denatured (Fig. 2, curve d). Not only was the absorption maximum of the $BR_{D96N}$ film obviously blue-shifted from 570 to 548 nm, but also a new photostate with an absorption maximum of about 370 nm appeared, which is similar to some photoproducts that were assumed by some authors to correspond to (more or less) released or dissociated retinal molecules.[22] It may therefore be concluded that $BR_{D96N}$ : diaza-15-crown-5 above a 1 : 150 ratio has a strong denaturing effect on the purple membrane. Analogously, in $BR_{WT}$–PVA films, when diaza-15-crown-5 was added as a chemical additive, the same blue shifts of the absorption maximum were observed, but a $BR_{WT}$ : diaza-15-crown-5 molecular ratio of about 1 : 250 could be used without adversely affecting the spectral properties of the film.[19]

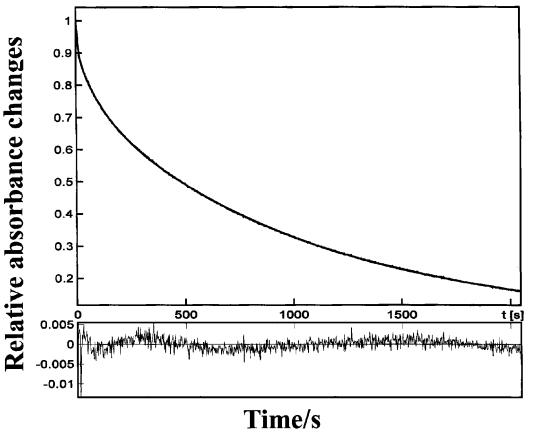



**Fig. 4** A typical example of a three-exponential fit to the measured M decay for a $BR_{D96N}$–PVA film with a $BR_{D96N}$ : diaza-15-crown-5 molecular ratio of 1 : 100.

### Kinetic measurements of the samples

The role of diaza-15-crown-5 in the M intermediate state decay has been investigated for the two types of BR. It is well known that the decay of the M state shows a complex multi-exponential behavior.[15] We have fit the M state decay for all of the chemically modified $BR_{WT}$–PVA and $BR_{D96N}$–PVA films at different diaza-15-crown-5 contents, in a least-squares fashion, to an equation of the form: $Y(t) = A\ [\exp(-t/\tau_1)] + B\ [\exp(-t/\tau_2)] + C\ [\exp(-t/\tau_3)]$. We assumed that $A + B + C = 1$ and $A$, $B$, and $C$ represented the relative amplitudes of the time constants $\tau_1$, $\tau_2$, and $\tau_3$, respectively. The results indicate that this three-exponential function gives satisfactory fits with sufficiently small residuals ($< 1\%$). A typical example of the fitting of a three-exponential curve to measurements of the M state decay for a $BR_{D96N}$–PVA film with $BR_{D96N}$ : diaza-15-crown-5 molecular ratio of 1 : 50 is presented in Fig. 4. The three relaxation time constants ($\tau_i$) and corresponding relative amplitudes ($A$, $B$, $C$) for each curve are summarized in Table 1.

This fit is similar to one described earlier,[23] but with more parallel M-like intermediates. These several, parallel M-like intermediates approximate a distribution of the substrate, with the protein dried in slightly different conformations. Somehow the diaza-15-crown-5 makes this distribution more heterogeneous. This makes it necessary to approximate the distribution with more parallel branches in the model fit.

As shown in Table 1, addition of diaza-15-crown-5 significantly slowed down the decay time of the M intermediate state in the $BR_{WT}$ and $BR_{D96N}$ films, and this prolongation effect increased gradually with increasing diaza-15-crown-5 concentration. Compared to the BR films without a chemical additive, the three time constants vary about 5- to 300-fold with 1 : 50–1 : 250 molecular ratios for both BR films of the WT and D96N mutant. These results indicate that diaza-15-crown-5 is an efficient chemical additive for both $BR_{WT}$ and $BR_{D96N}$.

The reason for this remarkable prolongation may originate from the following properties of the additive. Firstly, it is well known that increasing the pH of the environment of BR can increase the M state lifetime.[16] Since the –NH– group in the ring is strongly basic, the addition of diaza-15-crown-5 could

**Table 1** Time constants and coreesponding relative amplitudes of the three-exponential fits to the decay kinetics of the M state in $BR_{WT}$–PVA and $BR_{D96N}$–PVA films with different diaza-15-crown-5 concentrations. The relative amplitudes are given in brackets under the corresponding time constants

| | $\tau_1$/s | $\tau_2$/s | $\tau_3$/s |
|---|---|---|---|
| $BR_{WT}$ : diaza-15-crown-5 | | | |
| No additive[15] | $0.244 \pm 0.100$ | $1.25 \pm 0.4$ | $10.1 \pm 3.0$ |
| | [$0.30 \pm 0.06$] | [$0.42 \pm 0.04$] | [$0.28 \pm 0.06$] |
| 1 : 100 | $12.66 \pm 0.48$ | $116.5 \pm 1.4$ | $604.5 \pm 4.5$ |
| | [$0.339 \pm 0.002$] | [$0.308 \pm 0.005$] | [$0.351 \pm 0.003$] |
| 1 : 150 | $17.49 \pm 0.29$ | $208.6 \pm 1.7$ | $1111 \pm 6$ |
| | [$0.304 \pm 0.006$] | [$0.276 \pm 0.006$] | [$0.393 \pm 0.004$] |
| 1 : 200 | $22.47 \pm 0.26$ | $296.6 \pm 1.9$ | $1674 \pm 12$ |
| | [$0.277 \pm 0.004$] | [$0.283 \pm 0.007$] | [$0.429 \pm 0.006$] |
| 1 : 250 | $27.41 \pm 0.24$ | $382.1 \pm 2.3$ | $2298 \pm 15$ |
| | [$0.252 \pm 0.005$] | [$0.247 \pm 0.004$] | [$0.486 \pm 0.008$] |
| $BR_{D96N}$ : diaza-15-crown-5 | | | |
| No additive | $8.35 \pm 0.17$ | $93.93 \pm 0.39$ | $415.5 \pm 2.4$ |
| | [$0.351 \pm 0.009$] | [$0.306 \pm 0.003$] | [$0.344 \pm 0.006$] |
| 1 : 50 | $29.56 \pm 0.32$ | $334.6 \pm 1.7$ | $1740 \pm 7$ |
| | [$0.208 \pm 0.003$] | [$0.258 \pm 0.001$] | [$0.510 \pm 0.002$] |
| 1 : 75 | $35.39 \pm 0.37$ | $418.4 \pm 2.5$ | $2335 \pm 11$ |
| | [$0.142 \pm 0.005$] | [$0.228 \pm 0.003$] | [$0.605 \pm 0.005$] |
| 1 : 100 | $40.19 \pm 0.28$ | $497.1 \pm 2.9$ | $3026 \pm 14$ |
| | [$0.135 \pm 0.002$] | [$0.180 \pm 0.005$] | [$0.682 \pm 0.004$] |
| 1 : 150 | $45.26 \pm 0.37$ | $577.1 \pm 3.8$ | $3661 \pm 19$ |
| | [$0.130 \pm 0.008$] | [$0.154 \pm 0.006$] | [$0.686 \pm 0.007$] |

have the same effect as added base on the prolongation process. This effect was especially obvious in a $BR_{D96N}$ suspension with diaza-15-crown-5. To fit the M state decay for all of these suspension mixtures, a two-exponential function: $Y(t) = A[\exp(-t/\tau_1)] + B[\exp(-t/\tau_2)]$ was required (fitting residuals $< 1\%$); the fitting results are summarized in Table 2. Compared with the fitting results for $BR_{D96N}$–PVA films (Table 1), one component is removed due to the absence of the dehydration process. As shown in Table 2, since the pH of the suspensions increased gradually with additive concentration, the relaxation time constants ($\tau_i$) of the M state decay with diaza-15-crown-5 increased gradually. Compared to the $BR_{D96N}$ suspension without the additive, the fitting results changed only slightly, which indicates that diaza-15-crown-5 can play a similar role as base on the prolongation.

Secondly, the diaza-15-crown-5 additive has strong complexing properties towards $H^+$,[24] thus the number of protons available for recapture by the M state of BR molecules in BR-PVA films so that can relax back to the B state is reduced, and the rate of reprotonation of the Schiff base is significantly decreased. Thirdly, similar to the effect of triethanolamine on the BR photocycle,[18] the diaza-15-crown-5 group in suspension is hydrated and binds a proton. When water is removed by drying the samples, the crown ether loses its hydration shell and, by gaining a partial negative charge, it can play the role of a proton acceptor, if it penetrates in the proton channel of BR. In the dried samples, the negative electron pair of the diaza-15-crown-5 molecule binds a proton and changes the membrane surface charge, shifting the $pK_a$ of Asp-85 and the Schiff base in such a way that the proton acceptor transiently holds the proton for a longer time. It was shown earlier[23] that the decay of the M intermediate involves the protonation of the Schiff base by taking back a proton from Asp-85. In this case, the back-motion of the proton happens from a more negative acceptor complex, on a much slower time scale, as the protein relaxes to its initial state.

Furthermore, from Table 1 it can also be concluded that the effect of diaza-15-crown-5 on the $BR_{D96N}$–PVA film is larger than on the $BR_{WT}$–PVA film. At the same molecular ratio, M state decay time constants of $BR_{D96N}$ films are 2–5 times larger than those of $BR_{WT}$ films. For example, at a BR : diaza-15-crown-5 ratio of 1 : 100, the $\tau_1$, $\tau_2$, and $\tau_3$ of the $BR_{D96N}$–PVA film respectively reached 40.19, 497.1, and 3026 s, which are 3–5 times larger than the corresponding times of 12.66, 116.5, and 604.5 s in a $BR_{WT}$–PVA film. Even at the highest additive content ($BR_{WT}$ : diaza-15-crown-5 molecular ratio of 1 : 250) in a $BR_{WT}$–PVA film, the three time constants are still smaller than those of the $BR_{D96N}$ film, although for the latter the highest additive content level was 1 : 150 molecular ratio of $BR_{D96N}$ : diaza-15-crown-5. As is known, in $BR_{D96N}$, the interior proton donor, Asp-96, is replaced by Asn-96, which has lost the protonation function. Thus, in the decay of the M intermediate state, the process of the proton translocation step from Asp-96 to the Schiff base is absent, and the deprotonated Schiff base is directly reprotonated from the medium. Since diaza-15-crown-5 has strong basic and $H^+$ complexing properties, the number of protons in the medium is also reduced, thus the M state lifetime in the diaza-15-crown-5 enhanced $BR_{D96N}$–PVA film is significantly longer than that in the $BR_{WT}$ film.

**Table 2** Time constants and corresponding relative amplitudes (in brackets) of the two-exponential fits to the decay kinetics of the M state in $BR_{D96N}$ suspension with different diaza-15-crown-5 concentrations

| $BR_{D96N}$ : diaza-15-crown-5 | $\tau_1$/s | $\tau_2$/s |
|---|---|---|
| No additive | $40.71 \pm 0.28$ | $124.2 \pm 0.5$ |
| | $[0.417 \pm 0.009]$ | $[0.588 \pm 0.007]$ |
| 1 : 50 | $37.59 \pm 0.22$ | $101.7 \pm 0.4$ |
| | $[0.510 \pm 0.003]$ | $[0.496 \pm 0.005]$ |
| 1 : 100 | $38.83 \pm 0.23$ | $104.4 \pm 0.4$ |
| | $[0.425 \pm 0.007]$ | $[0.581 \pm 0.006]$ |
| 1 : 150 | $38.43 \pm 0.30$ | $108.3 \pm 0.5$ |
| | $[0.458 \pm 0.003]$ | $[0.558 \pm 0.004]$ |

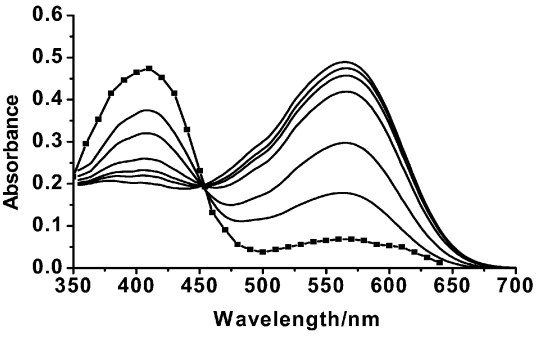



**Fig. 5** Photochromic property of a $BR_{D96N}$–PVA film with a $BR_{D96N}$ : diaza-15-crown-5 molecular ratio of 1 : 150 (from top to bottom at 410 nm, the curves correspond to times of 0 min, 3.5 min, 10 min, 30 min, 60 min, 90 min and 3 h, respectively after photoexcitation).

### Photochromic property

From Table 1, it can be seen that the 1 : 150 molecular ratio of $BR_{D96N}$ : diaza-15-crown-5 is the optimal one, and the BR film has the longest lifetime at this diaza-15-crown-5 concentration. This indicates that by a combination of genetic engineering and using chemical additives, the M state lifetime can be prolonged to longer times than those obtained by using either of these two methods. Fig. 5 shows the photochromic property of this $BR_{D96N}$–PVA film. The spectra were obtained by recording the subsequent absorption spectra as the M state relaxed back to the B state after 30 s photoexcitation with 560 nm light to reach a high accumulation of the M state. The zero time spectrum was obtained indirectly from the time-dependence of the absorbance monitored at different wavelengths. As shown in Fig. 5, an isosbestic point was observed at 452 nm, and the M state was not yet completely depleted even after one and a half hours. In addition, the photochromism between the M (yellow ) and B states (violet) could also be observed visually. Thus, the present $BR_{D96N}$–PVA film with a $BR_{D96N}$ : diaza-15-crown-5 molecular ratio of 1 : 150 provides significant potential for BR optical applications even under ambiant conditions.

## Conclusions

The lifetimes of the M intermediate state ($\tau_{1/e}$) for both $BR_{WT}$ and $BR_{D96N}$ in films have been significantly prolonged by using diaza-15-crown-5, which indicates that it is an effective chemical additive. Furthermore, by combination of genetic engineering and using a chemical additive, the M state lifetime can be prolonged to longer times than those obtained using either of these two methods. At the optimal $BR_{D96N}$ : diaza-15-crown-5 molecular ratio of 1 : 150, the photochromism time of a $BR_{D96N}$ film lasts about one and a half hours. This long-lived M state can be utilized to expand the use of BR materials in optical applications. The reason for this significant prolongation may originate from the strong basic and $H^+$ complexing properties of diaza-15-crown-5 and the change of the membrane surface charge caused by this additive. Further investigations on the reversibility of this chemically enhanced BR–PVA film are now in process.

## Acknowledgement

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