Thr90 is a key residue of the bacteriorhodopsin proton pumping mechanism

Alex Perálvarez[a], Ramon Barnadas[a], Manuel Sabés[a], Enric Querol[b], Esteve Padrós[a,*]

[a] *Unitat de Biofísica, Departament de Bioquímica i de Biologia Molecular, Facultat de Medicina, Universitat Autònoma de Barcelona, Bellaterra (Cerdanyola del Vallès), Barcelona 08193, Spain*

[b] *Institut de Biotecnologia i Biomedicina, Universitat Autònoma de Barcelona, Bellaterra (Cerdanyola del Vallès), Barcelona 08193, Spain*

Received 17 August 2001; revised 23 October 2001; accepted 23 October 2001

First published online 6 November 2001

Edited by Maurice Montal

Abstract Mutation of Thr90 to Ala has a profound effect on bacteriorhodopsin properties. T90A shows about 20% of the proton pumping efficiency of wild type, once reconstituted into liposomes. Mutation of Thr90 influences greatly the Schiff base/Asp85 environment, as demonstrated by altered λ_{max} of 555 nm and pK_a of Asp85 (about 1.3 pH units higher than wild type). Hydroxylamine accessibility is increased in both dark and light and differential scanning calorimetry and visible spectrophotometry show decreased thermal stability. These results suggest that Thr90 has an important structural role in both the unphotolysed bacteriorhodopsin and in the proton pumping mechanism. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Bacteriorhodopsin; Proton pumping; Helical kink; Conformational change; Hydrogen bonding; Thermal stability

1. Introduction

In the proton transport mechanism of bacteriorhodopsin (BR), two interrelated events have been recognized as fundamental: protonation/deprotonation reactions of some key amino acids and the Schiff base, and conformational changes [1–3]. The presence of conformational changes in BR during proton transport has been clearly described in the last years [1–9], but the structural basis of these movements is not still clear. The conformational changes that originate from the retinal after photoisomerization may be the result of the consecutive retinal changes and/or protonation changes, or may evolve independently of the retinal after the first event. Three Pro residues located near the middle of the helices B (Pro50), C (Pro91) and F (Pro186) could function as hinges facilitating these movements [10–14]. Especially, movement of the cytoplasmic end of helix F representing a transient opening of the cytoplasmic side may be important for Asp96 reprotonation [15–18]. Movements of the cytoplasmic loops may also be important for proton transfer in the cytoplasmic side.

It has been described recently that Ser and Thr residues bend α helices by forming an additional hydrogen bond with the i-3 or i-4 peptide carbonyl oxygen [19]. This effect could become important for helix C that carries the sequence Thr89-Thr90-Pro91 and several key side chains of the proton transport mechanism (Arg82, Asp85, Asp96). The importance of Thr89 has been pointed out in several papers [20–22], but Thr90 has so far received little attention. In addition to the possible induction of a bend in the helix, another interesting aspect of Thr90 is suggested by the recent three-dimensional structures of BR, which reveal the formation of an hydrogen bond between Thr90 (helix C) and Asp115 (helix D) [23–25]. Studies of model transmembrane helices [26] indicate that this may be a strong hydrogen bond. We describe in this work several properties of T90A that suggest an important structural role of Thr90 in the proton transport mechanism.

2. Materials and methods

The T90A mutant was obtained as in the previously described procedure [27]. The membrane was grown and purified by the standard method [28], and the mutation was confirmed from *Halobacterium salinarum* transformants by sequencing the *bop* gene from isolated DNA.

Deionized membranes were obtained after a 6-h dialysis against Dowex 50 W cation exchange resin. pH titrations were carried out by adding micro-volumes of HCl or NaOH solutions to membrane suspensions (1.5×10^{-5} M BR). To avoid contamination, pH adjustment of deionized samples was done by using duplicates. Absorption spectra were taken using an integrative sphere device (to minimize the lose of signal caused by light scattering) placed in a Varian Cary 3 spectrophotometer. Absorbance changes at 615 nm as a function of pH were used to monitor the purple-to-blue transition. Experimental data were normalized to the largest value at 615 nm and fitted to the Henderson–Hasselbach equation.

Differential scanning calorimetry (DSC) experiments were performed using a MicroCal MC2 instrument (MicroCal Inc, USA). Samples were dialyzed previously against water adjusted to pH 7.0, giving a final protein concentration of 1.5–2 mg/ml. Experiments were done under a nitrogen pressure of 1.7 atm to avoid sample evaporation at high temperatures. Scanning speed was set at 1.5 K/min. Three consecutive thermograms were registered for each sample. The first informs about the heat released or taken by the protein upon temperature increase. After cooling down to room temperature, a second and a third thermogram were run to check the reversibility of the transitions. Two corrections were applied to the first thermogram: (a) subtraction of the second thermogram, that acts as a blank and (b) subtraction of the chemical baseline using the method of Takahashi and Sturtevant [29]. T_m was defined as the temperature where the C_p value is maximal.

Analysis of thermal stability of dark-adapted T90A samples in H_2O (0.75×10^{-5} M) was carried out by recording absorption spectra in the UV-visible range during thermal ramps of 5°C steps, from 20 to 95°C in a Cary Bio3 spectrophotometer. Samples were allowed to stabilize for 8 min at each temperature.

Membrane suspensions (1.5×10^{-5} M BR) were reacted with 1 M

*Corresponding author. Fax: (34)-9-35811907.
E-mail address: esteve.padros@uab.es (E. Padrós).

Abbreviations: BR, bacteriorhodopsin; DSC, differential scanning calorimetry; EPC, egg yolk phosphatidylcholine

0014-5793/01/$20.00
PII: S0014-5793(01)03080-0

hydroxylamine in a medium containing 150 mM sodium phosphate (pH 7.0). Reactions under light were done using white light of 300 lux of luminance.

For liposome preparation, egg yolk phosphatidylcholine (EPC) was purified as described by Singleton et al. [30] and stored in chloroform solution at −20°C. Initial proteoliposome suspensions were obtained by adding the required amount of purple membrane suspension in 150 mM KCl, pH 7.0 to a dry film of EPC obtained by rotatory evaporation, and vortexing for 10 min. In all cases EPC concentration was 6 mg/ml and the lipid-to-BR ratio was 50:1 (w/w). Small proteoliposomes were obtained by high-pressure homogenization [31] with a Microfluidizer 110S (Newton, USA).

Liposome size distribution was measured by dynamic light scattering using a Microtrac Ultrafine Particle Analyser 150 spectrometer (Montgomeryville, USA). Samples were diluted with their aqueous medium in order to obtain a satisfactory signal. Data acquisition time was 10 min.

All proton pumping pH measurements were done in dim red light at 25°C. In order to avoid any pH gradient across the vesicle bilayers, the suspensions were kept in darkness for at least 30 min prior to pH measurements. After complete pH stabilization, the sample was illuminated with yellow-filtered light of a luminance of about 8×10^4 lux on the sample. The initial rate of proton pumping was determined by a linear fit of the pH changes within the first 10 s of illumination.

3. Results

The first evidence of the perturbed behavior of T90A is the absorption maximum, 555 nm in the light-adapted state (see Fig. 1A) as compared to 568 nm of wild type; this gives rise to a more reddish color for the mutant. Upon acidification, T90A shows a purple-to-blue transition similar to wild type (see Fig. 1C), although this mutant never reaches the λ_{max} of 603 nm in any condition analyzed. In the case of deionized sample, a double-peaked absorption spectrum is obtained at acid pH (maximum at 500 nm and shoulder at 592 nm; see Fig. 1B), indicative of two populations. At neutral pH, the spectrum presents a unique peak at 535 nm. Difference spectra of the sample at acid pH minus the sample at neutral pH show two positive bands at 620 and 445 nm, corresponding the blue and red forms (Fig. 1D). The λ_{max} of 535 nm at neutral pH contrasts with deionized wild type, which has a λ_{max} of 558 nm.

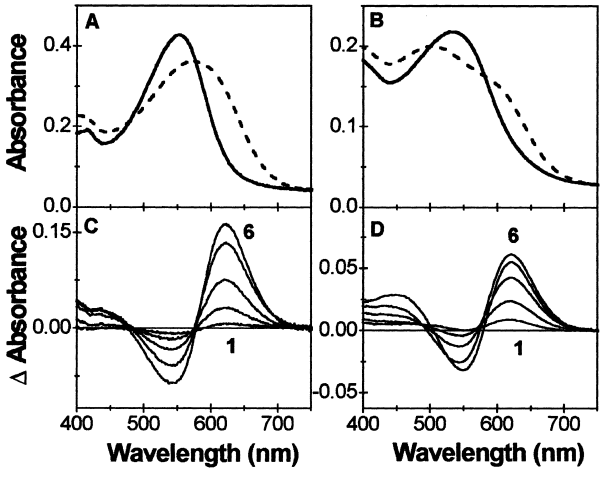

Fig. 1. Purple-to-blue transition of dark-adapted T90A. A: Absorption spectra of T90A in 150 mM KCl at pH 7.2 (solid line) and 2.0 (dashed line). B: Absorption spectra of deionized T90A at pH 7.0 (solid line) and 3.6 (dashed line). C: Curves 1–6 are the difference spectra ($pH_i - pH_{7.2}$) of T90A in 150 mM KCl, where pH_i values are 7.2, 6.2, 5.0, 4.1, 3.5 and 3.2. D: Curves 1–6 are the difference spectra ($pH_i - pH_{8.0}$) of deionized T90A, where pH_i values are 7.0, 6.4, 5.9, 5.1, 4.3 and 3.6.

Table 1
Apparent pK_a of Asp85 (the purple-to-blue transition), as obtained from the plot of absorption changes as a function of pH

	pK_a	
	Wild type	T90A
Deionized sample	5.5	6.3
H_2O	3.2	4.6
150 mM KCl	2.7	4.0

Table 1 shows the apparent Asp85 pK_a values, obtained from the plots of absorbance differences at 615 nm. In all the conditions analyzed, these values are higher than those of wild type, revealing that removal of the O-H group of Thr90 affects the Asp85 environment.

The hydroxylamine accessibility to the Schiff base in T90A mutant is increased as compared to wild type (Fig. 2). Under illumination, T90A has a $t_{1/2}$ of about 53 min as compared to 180 min for wild type. In the dark, T90A also shows enhanced accessibility ($t_{1/2}$ of about 4.5 h as compared to 160 h for wild type).

To study the role of Thr90 in the maintenance of BR structure, DSC experiments were performed. Thermograms of wild type and T90A in water at neutral pH are shown in Fig. 3, after correction with instrumental and chemical baselines. Important differences in both transitions are revealed. The pretransition, which reflects the cooperative disorganization of the hexagonal para-crystalline arrangement, shows a transition around 54°C, as compared to wild type at about 82°C. The main transition, that is a consequence of the cooperative denaturation of the tertiary structure, has a T_m of 83°C, as compared to 98°C for wild type. This indicates an easier loss of the inter-helical interactions upon temperature increase.

Changes in the visible absorption band also give information about protein denaturation, detected in this case through retinal release. Similar to wild type, when the T90A membrane suspensions are gradually heated, first appears the blue form (630 nm), followed by the appearance of the red form (approximately 460 nm) and finally, the free retinal. In

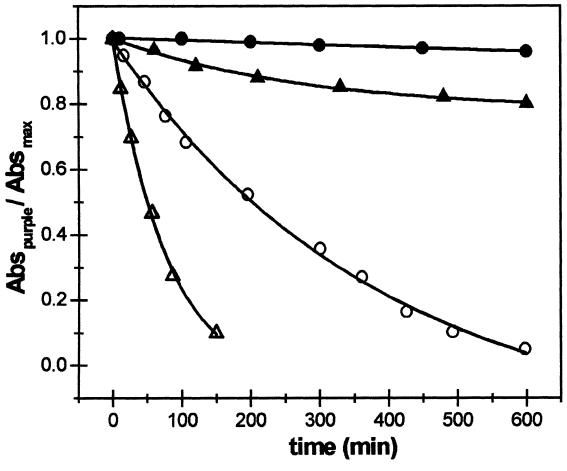

Fig. 2. Rate of hydroxylamine reaction with wild type BR (circles) and T90A (triangles) as measured by the absorption change in the visible. The reactions were carried out in dark (filled symbols) or under illumination with light of 300 lux (open symbols).

accordance with DSC results, thermal stability experiments of T90A show a lower temperature of retinal release, about 85°C in H_2O (pH 7.0), as compared to 90°C for wild type (data not shown).

In order to estimate proton transport efficiency, wild type BR and T90A were incorporated into liposomes using the high-pressure homogenization method, which gives a good reproducibility [31]. Fig. 4 presents pH changes as a function of time of illumination, in 150 mM KCl. The initial pumping rate of T90A is about 16% of that of wild type, and the photo steady pumping amount is about 20% of wild type. Size measurements showed that both preparations had a similar liposome size distribution, with a mean diameter of about 60 nm. The degree of orientation of BR into the liposomes was checked by papain digestion under conditions that cut the C-terminal tail, followed by electrophoresis (data not shown). No significant differences were found between wild type and the T90A mutant.

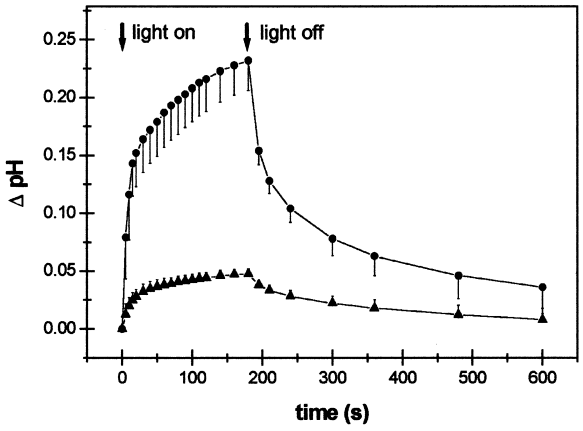

Fig. 4. Proton pumping rates of wild type BR (circles) and T90A (triangles) incorporated into liposomes, as a function of time. Data are the mean values from three independent samples. The bars represent half of the corresponding standard error. The initial pumping rate of T90A was about 16% of that of wild type, and the photo steady pumping amount, about 20%.

4. Discussion

One important result of this work is that the T90A mutant has between 16 and 20% of the wild type proton pumping efficiency, as evidenced by the small pH changes appearing upon illumination of proteoliposome suspensions. On the other hand, the altered values of λ_{max} and of pK_a of Asp85 in the resting state argue that the Asp85/Schiff base environment is distorted by the mutation. According to the published structural models of BR, Thr90 cannot interact directly with either the Schiff base, or with Asp85, or with water molecules. Yet the Cγ of Thr90 is in Van der Waals contact with C_{11}-C_{12} of the retinal [24,25], and the disappearance of this steric interaction could explain some of the effects observed. This could be similar to the steric interaction existing between Leu93 and the 13-methyl group of the retinal [32]. However, due to the more lateral location of Thr90 with respect to the retinal, as compared to Leu93, we favor the view that the changes induced by the mutation are due mainly to a structural influence achieved through two different and perhaps complementary interactions of Thr90: the strong hydrogen bond formed with Asp115 (with a distance of 2.40–2.50 Å, according to [23–25]) and the hydrogen bond formed with the peptide carbonyl oxygen of Trp86 (distance of about 2.55 Å). These strong hydrogen bonds may constitute key interactions for the maintenance of the correct location and bending of helix C. Even if hydrophobic interactions and water-mediated hydrogen bonds can facilitate the correct location of this helix, most likely these interactions are too weak to maintain the arrangement of helix C by themselves. In contrast, hydrogen bonding between transmembrane helices (in this case between helices C and D) is of strong nature [26]. Other hydrogen bonding inter-helical interactions involving helix C can also contribute to its stabilization, like Thr46 with Asp96, Tyr79 with Arg7 and Glu9, and Tyr83 with Trp189 [25]. However, the hydrogen bonds Thr90–Asp115 and Thr46–Asp96 are located near the middle of the helix and presumably will contribute the most to the stabilization of helix C.

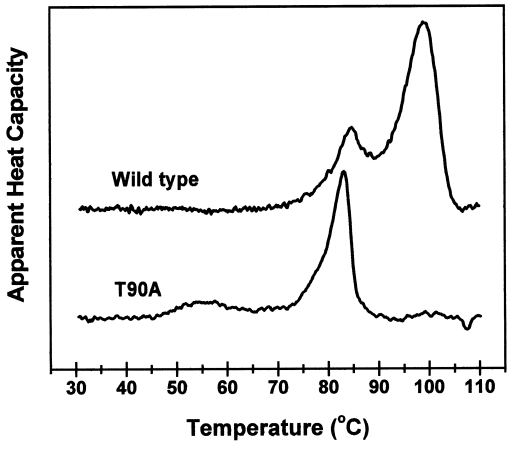

Fig. 3. DSC thermograms of wild type and T90A. The membranes were suspended in H_2O at 1.5–2.0 mg/ml, pH 7.0. All curves were corrected with the instrumental and chemical baselines. Scans were taken at 1.5 K/min.

It is likely that disappearance of the hydrogen bonds formed by the O-H group of Thr90 is responsible of the effects of the mutation. For example, rupture of the inter-helical hydrogen bond formed with Asp115 is in keeping with both the lower thermal stability of the mutant and the increased hydroxylamine accessibility. The main DSC transition, which is due to the destruction of tertiary interactions between helices including the retinal [33], is about 15°C lower than wild type; a lower T_m for retinal release is also obtained from visible spectroscopy. Similarly, the decrease of about 28°C of the DSC pre-transition argues that disappearance of the interactions maintained by Thr90 decreases the compact state of the protein and confers a less stable para-crystalline arrangement. In this context, the alteration of the infrared absorbance band of Asp115 giving rise to a signal in the difference spectra of photocycle intermediates of wild type [34,35] may be mainly a reflex of the strain induced over the hydrogen bond by the movement of the helices during the photocycle.

Interestingly, Trp86, Thr90 and Asp115 are highly conserved residues among homologous proteins from several halobacterial strains, giving support to the view that they are structurally important residues. Indeed, the homologous residue in halorhodopsin, Thr116, also forms hydrogen bonds with Asp141 (homologous to Asp115) in helix D and with the peptide carbonyl oxygen of Trp112 (homologous to

Trp86), as revealed by the recent high-resolution structure ([36]; PDB entry 1E12). Although at this time we can only speculate about the exact role of the Thr90–Asp115 and Thr90–Trp86 interactions, a possibility is that the Thr90–Asp115 link acts like a turning point, thus allowing the movement of helix C in the transport process, as has been readily detected by electron paramagnetic resonance spectroscopy [5] and X-ray diffraction [24]. It may be that lack of this interaction impels helix C to adopt a too separated location from the other helices, thus preventing the correct environments and interactions for the key side chains that are located in it (Arg82, Asp85, Asp96). On the other hand, Thr90 is central in the sequence Thr89-Thr90-Pro91 and this sequence has been described as capable of inducing kinks in helices [19]. The BR structural models effectively show a kink in helix C at the level of these amino acids. Possibly, a completely regular α helix could not form correctly the retinal cavity. This type of kink has also been involved in the conformational equilibrium between active and inactive states of G-protein coupled receptors [19,37].

Acknowledgements: We thank L. Pardo for suggesting the possible structural importance of Thr90, T. Lazarova and M. Márquez for helpful discussions, R. Needleman for his generous gift of the plasmid *pXL*NovR and *pSI-bop*, containing the *bop* gene, and E. Serrano and Y. Moreno for their skilful technical assistance. This work was supported by the Dirección General de Investigación, MCYT (Grant BMC2000-0121 and fellowship FP2000-6326) and the Direcció General de Recerca, DURSI (Grant 1999SGR-102).

References

[1] Balashov, S.P. (2000) Biochim. Biophys. Acta 1460, 75–94.
[2] Luecke, H., Schobert, B., Cartailler, J.P., Richter, H.T., Rosengarth, A., Needleman, R. and Lanyi, J.K. (2000) J. Mol. Biol. 300, 1237–1255.
[3] Subramaniam, S., Linddahl, M., Bullough, P., Faruqi, A.R., Tittor, J., Oesterhelt, D., Brown, L., Lanyi, J. and Henderson, R. (1999) J. Mol. Biol. 287, 145–161.
[4] Radzwill, N., Gerwert, K. and Steinhoff, H.J. (2001) Biophys. J. 80, 2856–2866.
[5] Rink, T., Pfeiffer, M., Oesterhelt, D., Gerwert, K. and Steinhoff, H.J. (2000) Biophys. J. 78, 1519–1530.
[6] Steinhoff, H.J., Savitsky, A., Wegener, C., Pfeiffer, M., Plato, M. and Möbius, K. (2000) Biochim. Biophys. Acta 1457, 253–262.
[7] Oka, T., Kamikubo, H., Tokunaga, F., Lanyi, J.K., Needleman, R. and Kataoka, M. (1999) Biophys. J. 76, 1018–1023.
[8] Mollaaghababa, R., Steinhoff, H.-J., Hubbell, W.L. and Khorana, H.G. (2000) Biochemistry 39, 1120–1127.
[9] Oka, T., Yagi, N., Fujisawa, T., Kamikubo, H., Tokunaga, F. and Kataoka, M. (2000) Proc. Natl. Acad. Sci. USA 97, 14278–14282.
[10] Gerwert, K., Hess, B. and Engelhard, M. (1990) FEBS Lett. 261, 449–454.
[11] Deber, C.M., Sorrell, B.J. and Xu, G.Y. (1990) Biochem. Biophys. Res. Commun. 172, 862–869.
[12] Sankararamakrishnan, R. and Vishveshwara, S. (1993) Proteins 15, 26–41.
[13] Lu, H., Marti, T. and Booth, P. (2001) J. Mol. Biol. 308, 437–446.
[14] Ludlam, C.F., Sonar, S., Lee, C.P., Coleman, M., Herzfeld, J., RajBhandary, U.L. and Rothschild, K.J. (1995) Biochemistry 34, 2–6.
[15] Kamikubo, H., Kataoka, M., Varo, G., Oka, T., Tokunaga, F., Needleman, R. and Lanyi, J.K. (1996) Proc. Natl. Acad. Sci. USA 93, 1386–1390.
[16] Koch, M.H., Dencher, N.A., Oesterhelt, D., Plohn, H.J., Rapp, G. and Buldt, G. (1991) EMBO J. 10, 521–526.
[17] Subramaniam, S., Gerstein, M., Oesterhelt, D. and Henderson, R. (1993) EMBO J. 12, 1–8.
[18] Vonck, J. (1996) Biochemistry 35, 5870–5878.
[19] Ballesteros, J.A., Deupi, X., Olivella, M., Haaksma, E.E.J. and Pardo, L. (2000) Biophys. J. 79, 2754–2760.
[20] Kandori, H., Kinoshita, N., Yamazaki, Y., Maeda, A., Shichida, Y., Needleman, R., Lanyi, J.K., Bizounok, M., Herzfeld, J., Raap, J. and Lugtenburg, J. (1999) Biochemistry 38, 9676–9683.
[21] Kandori, H., Yamazaki, Y., Shichida, Y., Raap, J., Lugtenburg, J., Belenky, M. and Herzfeld, J. (2001) Proc. Natl. Acad. Sci. USA 98, 1571–1576.
[22] Russell, T.S., Coleman, M., Rath, P., Nilsson, A. and Rothschild, K.J. (1997) Biochemistry 36, 7490–7497.
[23] Essen, L.-O., Siegert, R., Lehmann, W.D. and Oesterhelt, D. (1998) Proc. Natl. Acad. Sci. USA 95, 11673–11678.
[24] Royant, A., Edman, K., Ursby, T., Pebay-Peroula, E., Landau, E.M. and Neutze, R. (2000) Nature 406, 645–648.
[25] Luecke, H., Schobert, B., Richter, H.T., Cartailler, J.P. and Lanyi, J.K. (1999) J. Mol. Biol. 291, 899–911.
[26] Zhou, F.X., Cocco, M.J., Russ, W.P., Brunger, A.T. and Engelman, D.M. (2000) Nat. Struct. Biol. 7, 154–160.
[27] Sanz, C., Lazarova, T., Sepulcre, F., González-Moreno, R., Bourdelande, J.-L., Querol, E. and Padrós, E. (1999) FEBS Lett. 456, 191–195.
[28] Oesterhelt, D. and Stoeckenius, W. (1974) Methods Enzymol. 31, 667–678.
[29] Takahashi, K. and Sturtevant, J.M. (1981) Biochemistry 20, 6185–6190.
[30] Singleton, W.S., Gray, M.S., Brown, M.L. and White, J.L. (1965) J. Am. Oil Chem. Soc. 42, 53–57.
[31] Barnadas, R. and Sabés, M. (2001) Methods Enzymol., in press.
[32] Delaney, J.K., Schweiger, U. and Subramaniam, S. (1995) Proc. Natl. Acad. Sci. USA 92, 11120–11124.
[33] Cladera, J., Galisteo, M.L., Sabés, M., Mateo, P.L. and Padrós, E. (1992) Eur. J. Biochem. 207, 581–585.
[34] Maeda, A., Sasaki, J., Shichida, Y., Yoshizawa, T., Chang, M., Ni, B., Needleman, R. and Lanyi, J.K. (1992) Biochemistry 31, 4684–4690.
[35] Sasaki, J., Lanyi, J.K., Needleman, R., Yoshizawa, T. and Maeda, A. (1994) Biochemistry 33, 3178–3184.
[36] Kolbe, M., Besir, H., Essen, L.-O. and Oesterhelt, D. (2000) Science 288, 1390–1396.
[37] Ballesteros, J.A., Shi, L. and Javitch, J.A. (2001) Mol. Pharmacol. 60, 1–19.