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Purification of histidine tagged bacteriorhodopsin, *pharaonis* halorhodopsin and *pharaonis* sensory rhodopsin II functionally expressed in *Escherichia coli*

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Abstract Bacteriorhodopsin (BR) from *Halobacterium salinarum* as well as halorhodopsin (pHR) and sensory rhodopsin II (pSRII) from *Natronobacterium pharaonis* were functionally expressed in *E. coli* using the method of Shimono et al. [FEBS Lett. (1997) 420, 54–56]. The histidine tagged proteins were purified with yields up to 1.0 mg/l cell culture and characterized by ESI mass spectrometry and their photocycle. The pSRII and pHR photocycles were indistinguishable from the wild type proteins. The BR photocycle was considerably prolonged. pSOII is located in the cytoplasmic membrane and the C-terminus is oriented towards the cytoplasm as determined by immunogold labelling.

Key words: Bacterial rhodopsin; Phoborhodopsin; Photocycle; Eubacterial expression; Mass spectrometry; Immunogold labelling

1. Introduction

Bacteriorhodopsin (BR) from *Halobacterium salinarum*, halorhodopsin (pHR) and sensory rhodopsin II (pSRII) from *Natronobacterium pharaonis* belong to the family of seven helix membrane proteins which carry retinal as a chromophore. BR and pHR function as light driven ion pumps [1], whereas light absorption by pSRII mediates a repellent response of the bacteria [2,3]. The absorption maxima of BR and pHR are found at around 570 nm, that of pSRII at 497 nm with additional maxima of vibronic bands at 457 nm, 420 nm and 370 nm [4,5]. After absorption of a photon the all-*trans* retinylidene chromophore which is bound to a Lys residue via a protonated Schiff base isomerizes to a 13-*cis* configuration. In the succeeding sequence of thermal reactions which include charge transfer steps, *cis-trans* isomerization of retinal and conformational changes of the protein, the physiological tasks of the bacterial rhodopsins, namely vectorial ion transfer or signal transduction, are executed. For further information see [6–8].

Several attempts have been made to enable the heterologous expression of BR in *Escherichia coli*. Khorana and co-workers were able to express and purify retinal-free bacterioopsin (BO) [9]. After solubilization in SDS the opsin could be refolded into lipids and reconstituted to functionally active BR. An overexpression of functionally active BR was attempted by fusing BO to different signal sequences, however, these experiments also remained unsuccessful [10]. The first functional expression of an archaeal retinal protein (phoborhodopsin, pSRII) in *E. coli* was recently achieved by Shimono et al. [11].

Based on this latter work we describe the functional expression of histidine tagged BR (BR-His), pHR (pHR-His) and pSRII (pSRII-His) and their (one step) purification. Furthermore, the photocycle kinetics of the heterologously expressed proteins which have been reconstituted into lipid bilayers are compared with those of the wild type proteins and the cellular location and orientation of pSOII within the cytoplasmic membrane is investigated.

2. Materials and methods

2.1. Bacterial strains

As a host for DNA manipulations *E. coli* XL1 was used. Gene expression was carried out in *E. coli* BL21(DE3).

2.2. Construction of expression plasmids

Expression vectors were constructed using the plasmid pET27bmod derived from pET27b (Novagen, Madison, WI, USA) via deletion of the *Pel*B leader sequence [12], introducing a *Nco*I cloning site as the translational start. The genes of the three opsins BOP, pHOP and pSOPII were amplified by PCR using template plasmids containing the mature protein sequences in the case of BOP [13] and pHOP (Seidel, unpublished results) or the subcloned genomic *Sac*I/*Sal*I fragment in the case of pSOPII [14]. PCR was carried out using oligonucleotides introducing 5′-*Nco*I/3′-*Bam*HI restriction sites for BOP and pHOP and 5′-*Nco*I/3′-*Eco*RI for pSOPII, respectively. The stop codons were deleted during the amplification. The restricted PCR products were ligated into pET27bmod. Subsequently, the pET27 'opsin' plasmids produced were digested with *Bam*HI/*Hin*dIII in the case of BOP and pHOP and *Eco*RI/*Hin*dIII for pSOPII, respectively. In the next step, oligonucleotide pairs were ligated into the gaps introducing a sequence coding for a terminal histidine tag. This cloning strategy results in the following N- and C-terminal peptide sequences:

BOP: ^{1}MAQITG.....AAATS^{248}**GSHHHHHH**
HOP: ^{1}MAETLP.....TPADD^{291}**GSHHHHHH**
pSOPII: ^{1}MVGLTT.....AVADE^{241}**NSHHHHHHH**

For fusing the antigenic HSV-tag to pSRII, pSOPII was amplified as described above and ligated into the *Nco*I/*Eco*RI restricted vector pET27bmod.

DNA sequencing was carried out using the Dye Didesoxy kit from Applied Biosystems (CA).

2.3. Protein expression and purification

Transformed *E. coli* BL21(DE3) cells were grown at 37°C in 2TY medium supplemented with 50 μg/ml kanamycin in a 30 l fermenter. At an OD_{578} of 0.6–0.7, 1 mM IPTG and 10 μM all-*trans* retinal were added [11]. After an induction period of 2.5 h the cells were harvested, washed and resuspended in a Tris-HCl buffer (50 mM Tris-HCl, 5 mM $MgCl_2$, pH 8.0) and finally broken up in a microfluidizer (Micro-

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Abbreviations: BR, bacteriorhodopsin; PHR, *pharaonis* halorhodopsin; PSRII, *pharaonis* sensory rhodopsin II; PSOII, *pharaonis* sensory opsin II; ESI, electron spray ionization; IPTG, isopropyl-1-thio-β-galactoside; TFE, 2,2,2-trifluoroethanol

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fluidics Corporation, Newton, MA). Membranes were sedimented at 100 000 × *g* for 1 h at 4°C and solubilized in buffer S (1.5% DM, 300 mM NaCl, 50 mM MES, 5 mM imidazole, pH 6.0) for 16 h at 8°C. After centrifugation of the solubilized membranes (100 000 × *g*, 1 h, 4°C) the supernatant was incubated with Ni-NTA agarose (Qiagen, Hilden, Germany) for 1 h at 8°C with slow shaking of the vessel. The Ni-NTA resin was filled into a chromatography column and washed extensively with buffer W (0.15% DM, 300 mM NaCl, 50 mM MES, pH 6.0) with an increasing imidazole concentration (≤ 50 mM) to remove unspecifically bound proteins. Subsequently, the histidine tagged proteins were eluted in buffer E (0.05% DM, 300 mM NaCl, 50 mM Tris-HCl, 150 mM imidazole, pH 7.5).

2.4. Reconstitution of bacterial rhodopsins into polar lipids

For reconstitution of the solubilized bacterial rhodopsins into polar lipids isolated from *H. salinarum*, a thin film (15-fold molar excess, *M* = ca. 1000 g/mol) was prepared by dissolving the lipids in 1 ml of $CHCl_3$ and evaporating the solvent carefully in a stream of argon. BR, pHR or pSRII (1–2.5 mg each) dissolved in buffer E were added to the lipids and the suspension was gently stirred for 30 min at 30°C. The detergent was removed by dialysis against 5 l of 200 mM NaCl, 50 mM Tris-HCl, pH 7.5 (5× for 24 h at 8°C), followed by two dialysis steps against 2.5 l of a buffer which was also used in the photocycle experiments (BR-His: 150 mM NaCl, 20 mM NaH_2PO_4, pH 7.0; pHR-His: 150 mM NaCl, 0.62 M Na_2SO_4, 20 mM NaH_2PO_4, pH 6.0; pSRII-His: 150 mM NaCl, 10 mM Tris-HCl, pH 8.0). The reconstituted proteins were sedimented by centrifugation (150 000 × *g*, 16 h, 4°C) and resuspended in the final dialysis buffer.

2.5. ESI mass spectrometry

Mass spectra were recorded on a LCQ electrospray mass spectrometer (Finnigan MAT, USA) equipped with a nanospray source (H. Prinz, A. Lovin and M. Konrad, in preparation). The solubilized bacterial rhodopsins were prepared for mass spectrometry first by removing excess NaCl (repeated washing with 10 mM Tris-HCl in a centrifugal concentrator (MWCO 30 kDa)). In a second step samples were prepared according to the method of Hufnagel et al. [15] with the exception that a different solvent mixture ($CHCl_3$/MeOH/TFE/H_2O/HCOOH [100/75/50/35/4 (v/v)]) was used to dissolve the precipitated proteins. Mass spectrometric data were recorded in the range of 1000–2000 amu with an accuracy of 100 ppm. The average molecular masses of the proteins were calculated from *m/z* relations of multiple charged ions using the Finnigan deconvolution software.

2.6. Immunogold labelling and electron microscopy

E. coli BL21(DE3) transformed with pSOPIIHSVHis/pET27bmod were grown in 2TY medium supplemented with 50 μg/ml kanamycin to a final OD_{578} = 0.6 and then induced with 1 mM IPTG. The addition of retinal was omitted. The cells were fixated by a mixture of 4% paraformaldehyde and 0.2% glutaraldehyde and embedded in LR White (London Resin Company). The immunolabelling procedure was performed on ultrathin sections of embedded cells. For detection of pSOII HSV tag antibody (Novagen, Madison, WI, USA) was used. Binding sites of the primary antibody were visualized by 12 nm gold coupled IgG antibodies (Dianova, Hamburg, Germany). Images were recorded with a transmission electron microscope (Zeiss 902) at an acceleration voltage of 80 kV.

2.7. Flash spectroscopy

Flash photolysis and data treatment were done as outlined by Chizhov et al. [16]. Transient absorption changes were recorded at 20°C while varying the wavelengths from 390 nm to 710 nm (BR-His), 400 nm to 710 nm (HR-His) and 370 nm to 660 nm (pSRII-His) in intervals of 10 nm.

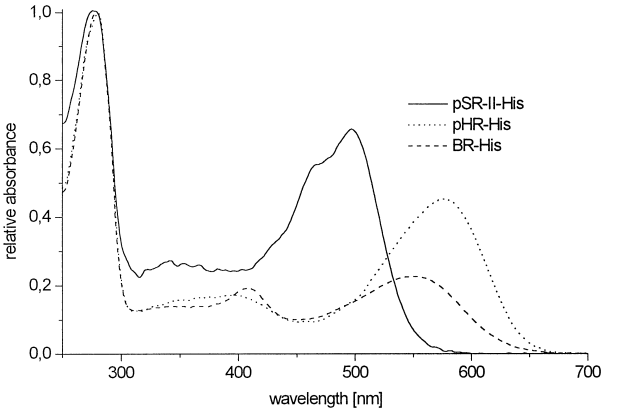

Fig. 1. Absorption spectra of purified pSRII-His, pHR-His and BR-His.

3. Results and discussion

3.1. Purification of the His tagged bacterial rhodopsins

The mature BR, pHR and pSRII genes were amplified from plasmids containing the sequences of the wild type proteins and ligated into the multiple cloning site of the *E. coli* expression vector pET27bmod together with oligonucleotides introducing a C-terminal histidine tag. Sequencing confirmed that no mutations occurred during PCR amplification. Protein expression was carried out as described by Shimono et al. [11]. The histidine tagged proteins were extracted from the solubilized membrane faction by incubation with Ni-NTA-agarose. The resin was filtered, washed and the bound protein eluted with a step gradient of imidazole.

The purified proteins were characterized by SDS-PAGE (data not shown), their absorption spectra (Fig. 1, see also Table 1 for biochemical and spectroscopic data) and their molecular mass. The absorption maxima of the retinylidene chromophore were identified at 552 nm for dark adapted BR-His, at 575 nm for pHR-His, and 497 nm pSRII-His and were found to be identical to those of the wild type proteins. The yields for BR-His, pHR-His and pSRII-His calculated from the optical density at λ_{max} were 84 μg, 0.64 mg and 1.0 mg per liter of cell culture, respectively. Only minor impurities (< 10%) were detected by SDS-PAGE analysis in the pHR and pSRII probes. However, BR-His could only be purified to a yield of about 60% with a major contaminant absorbing at around 408 nm. From its absorption spectra in the reduced

Table 1
Characterization of BR-His, pHR-His, and pSRII-His

Sample	Yield (μg/l cell culture)	Purity (%)	Molecular mass (determined[a])(Da)	Molecular mass (calulated) (Da)
BR-His	84	60	27 635[b]	27 639[b]
			27 793[c]	27 770[d]
pHR-His	640	> 90	31 852[b]	31 858[b]
			–[d]	31 989[d]
pSRII-His	1000	> 90	26 647[d]	26 645[d]

[a]Additional masses resulting from salt adducts (Na^+: +22 Da, PO_4^{2-}: +99 Da) are not listed (see text).
[b]N-terminal Met deletion.
[c]Sodium adduct.
[d]Unprocessed.

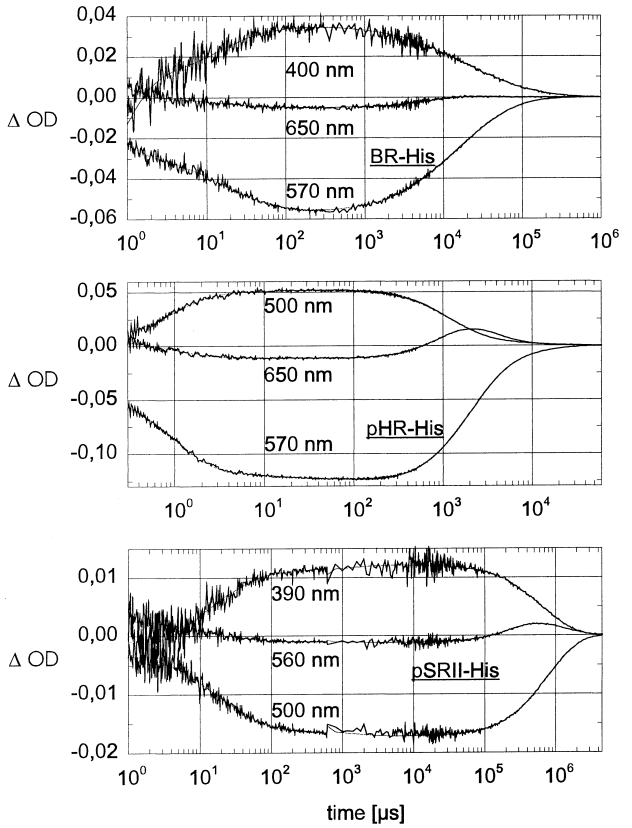

Fig. 2. Traces of transient absorption changes after photoexcitation of BR-His (upper panel), pHR-His (middle panel) and pSRII-His (lower panel). The time courses are shown only for the indicated wavelengths. The result of the multi-exponential global fit is represented by the thin solid lines.

and oxidized state (data not shown) it can be assigned to the Soret band of a cytochrome which is probably derived from *E. coli* membranes. The cytochrome contamination did not interfere with the photocycle measurements.

Although all three bacterial rhodopsins were cloned into the same vector with identical flanking regions, the yields of the purified proteins differed considerably. BR-His could only be isolated in amounts one order of magnitude less than pSRII-His. Dunn et al. [9] described relatively high expression yields for BO in *E. coli*. Assuming similar yields for BO-His its low recovery rate might be due to posttranslational precipitation and/or proteolytic degradation.

The molecular masses of the bacterial rhodopsins were verified by ESI mass spectrometry. The results are summarized in Table 1. The mass spectrum of the pSRII-His sample displays a dominant peak at 26 647 ± 2 Da which is close to the calculated mass of 26 645 Da. The additional peaks observed in this spectrum separated by 22 amu can be attributed to sodium adducts of the parent molecule.

Unfortunately, the molecular masses of BR-His and pHR-His could not be determined withe the same accuracy. BR-His formed multiple sodium adducts (BR-His*Na^+: 27 793 Da; calculated: 27 792 Da) but also phosphate associated with this molecule was identified. Each ionic species contributed similarly to the total sum of ions contained in the ion trap of the mass spectrometer thereby increasing the signal to noise ratio. In addition to the multiple sodium and phosphate adducts the BR-His sample consisted also of a posttranslationally modified species which lacked the N-terminal Met (observed: 27 635 Da; calculated: 27 639 Da).

The mass spectrum of the pHR-His probe displayed only a low signal amplitude. The major peak corresponded to a mass of 31 852 Da which can be attributed to pHR-His lacking the N terminal methionine (calculated: 31 858 Da). Additional sodium adducts were not observed, but an adduct peak at around 31 951 Da could be assigned to pHR-His*phosphate (calculated: 31 957 Da).

The mass spectrometric results revealed a different posttranslational processing of pHR-His and Br-His on the one hand and of pSRII on the other hand. According to Hirel et al. [17] the maximum side-chain length of the penultimate amino acid correlates with the probability of methionine excision from proteins expressed in *E. coli*. Likewise this can explain the different processing of the three bacterial rhodopsins which contain either Ala (BO-His and pHO, processed) or Val (pSOII, unprocessed) in the penultimate position.

3.2. Photocycle kinetics

For the analysis of the photocycle kinetics, the solubilized rhodopsins were reconstituted into polar lipids from *H. salinarum* to resemble a more natural protein environment. The transient absorption changes were recorded at wavelengths which cover the whole spectral range of the initial ground states and the corresponding intermediates. These data sets allowed for a detailed multi-exponential global fit [18,19]. Fig. 2 shows the transient absorption changes of BR-His, pHR-His and pSRII-His at selected wavelengths which are representative of the depletion and regeneration of the initial ground state (lower trace), and the formation and decay of characteristic intermediate states. For example, the upper trace (measuring wavelength 400 nm) of the panel, showing the transient absorption changes of BR-His, represents the formation and decay of the M intermediate which absorbs maximally at 400 nm. The multi-exponential global fit of the absorbance changes provided for the three pigments' apparent half-times which are – with the exception of BR-His –

Table 2
Apparent half-times of the photocycles of His tagged bacterial rhodopsins and their wild type counterparts

Protein	τ_1 [μs]	τ_2 [μs]	τ_3 [μs]	τ_4 [μs]	τ_5 [ms]	τ_6 [ms]	τ_7 [ms]	τ_8 [ms]
BR-His	0.5	8.5	38	n.r.[a]	0.6	5.3	24	117
Br-wt[16]	0.8	5.9	35	91	0.5	1.7	5	23
pHR-His	0.7	10	220	590	1.5	8.3		
pHR-wt[b]	0.4	50	250	450	1.5	20		
pSRII-His	0.9	12	51	940	74	150	440	735
pSRII-wt[5]	1	16	50	1500	120	300	500	1500

[a]Not resolved.
[b]I. Chizhov and M. Engelhard, unpublished results.

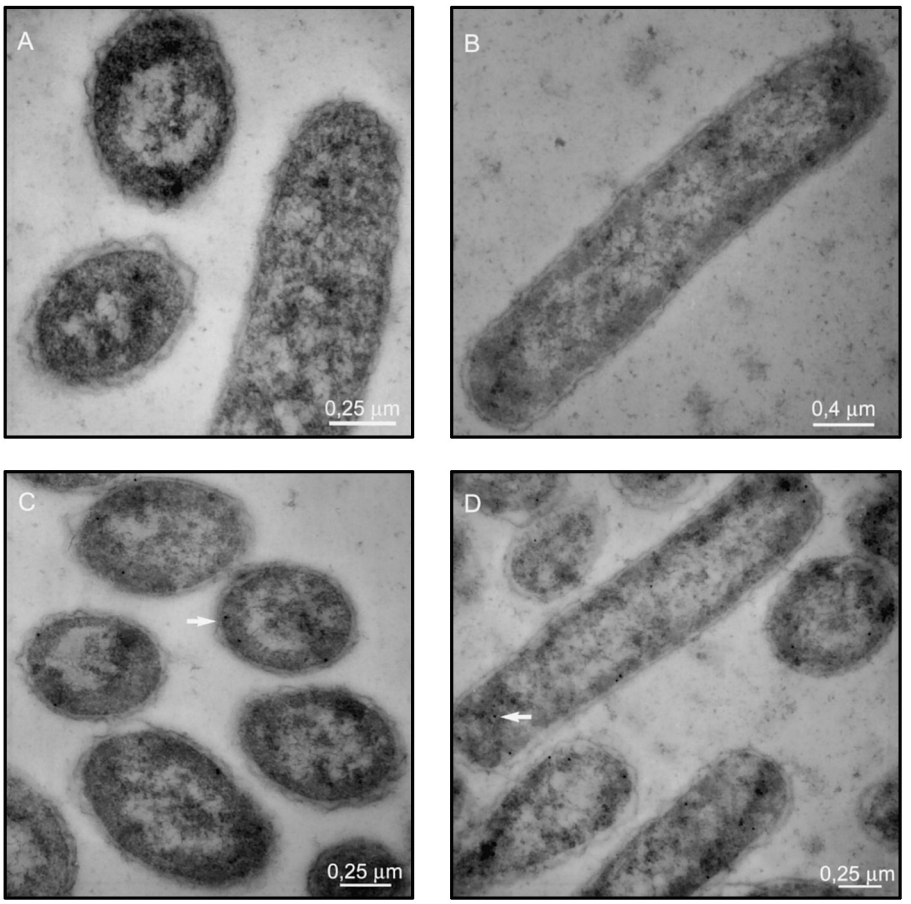

Fig. 3. Electron micrographs of ultrathin sections of *E. coli* pSOPIIHSVHis after immunogold labelling. A: Control 1: non-induced cells. B: Control 2: induced cells with secondary antibody. C and D: Induced cells with primary and secondary antibodies. The positions of representative colloidal gold particles on the inside of the cytoplasmic membrane (C) and in the cytoplasm (D) are indicated by arrows.

almost indistinguishable from those of the corresponding proteins expressed in their natural hosts (see Table 2) ([5,16]; I. Chizhov and M. Engelhard, unpublished results). The photocycle kinetics of BR-His differs in the later stages of the reformation of the initial ground state. The rate constants τ_6–τ_8 are slowed down by approximately a factor of five. The reason for these differences might be found in the poorer purity of the sample, the small amount available, and/or the C-terminal histidine tag which might interfere in the pick-up of the proton from the cytoplasm.

3.3. Cellular location of PSRII

To clarify the cellular location of the overexpressed archaebacterial receptors, immunogold labelling was performed on *E. coli* cells expressing pSOII with the HSV tag/His tag anchor. About 70 *E. coli* cells were analyzed. As a control uninduced cells were treated similarly and in a second control the primary antibody was omitted. Gold particles could be identified as sharp black spots in the recorded images (as examples two gold particles are marked by arrows in Fig. 3). The cellular localization of pSOII was determined by counting gold grains in all electron micrographs differentiating for cytoplasm (a), cytoplasmic membrane (b), periplasmic space (c), cell wall (d) and extracellular medium (e). This analysis revealed that gold particles could exclusively be detected in the cytoplasm (40%) and at the cytoplasmic side of the cellular membrane (60%) (Table 3). Supporting results were obtained from fractionation of the cells (data not shown). The observation that pSOII was not completely integrated into the cellular membrane might be caused by different factors. It is

Table 3
Localization of gold conjugates

	Non-induced cells (+prim. +sec. antibody) $n^a = 67$	Induced cells (+sec. antibody) $n^a = 22$	Induced cells (+prim. +sec. antibody) $n^a = 72$
Cytoplasm	8	1	114
Cytoplasmic membrane	2	0	160
Periplasm	0	0	3
Outer cell wall	0	0	2
Medium	1	1	0

[a] n = number of cells analyzed.

possible that the cytoplasmic fraction partially consists of misfolded species which do not integrate into the membrane or that the synthesis rate of pSOII is faster than its incorporation into the membrane.

Since the antibodies recognize the antigenic (C-terminal) HSV tag, the C-terminus of pSOII is oriented – like the bacterial rhodopsins in their natural environment – towards the cytoplasm. Further transport of pSOII into the periplasm or integration into the cell wall can be excluded because no gold particles were detected in these compartments. It has to be emphasized that the insertion into the cellular membrane occurs without fusion to a signal sequence.

In summary, the above experiments have shown that bacterial rhodopsins can functionally be expressed in the *E. coli* cytoplasmic membrane in an orientation which is identical to that of the pigments in their natural host. The modification of the C-termini by a His tag made it possible to isolate the proteins efficiently in high yields and purity. These results provide useful tools for the preparation of mutants, for the incorporation of isotope labelled amino acids, and for the modification of the proteins by retinal analogues.

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References

[1] Haupts, U., Tittor, J., Bamberg, E. and Oesterhelt, D. (1997) Biochemistry 36, 2–7.
[2] Takahashi, T., Tomioka, H., Kamo, N. and Kobatake, Y. (1985) FEMS Microbiol. Lett. 28, 161–164.
[3] Scharf, B., Pevec, B., Hess, B. and Engelhard, M. (1992) Eur. J. Biochem. 206, 359–366.
[4] Takahashi, T., Yan, B., Mazur, P., Derguini, F., Nakanishi, K. and Spudich, J.L. (1990) Biochemistry 29, 8467–8474.
[5] Chizhov, I., Schmies, G., Seidel, R., Sydor, J.R., Lüttenberg, B. and Engelhard, M. (1998) Biophys. J. 75, 999–1009.
[6] Lanyi, J.K. and Váró, G. (1995) Isr. J. Chem. 35, 365–385.
[7] Oesterhelt, D. (1995) Isr. J. Chem. 35, 495–513.
[8] Spudich, J.L. (1998) Mol. Microbiol. 28, 1051–1058.
[9] Dunn, R.J., Hacket, N.R., McCoy, J.M., Chao, B.H., Kimura, K. and Khorana, H.G. (1987) J. Biol. Chem. 262, 9246–9254.
[10] Karnik, S., Doi, T., Molday, R. and Khorana, H.G. (1990) Proc. Natl. Acad. Sci. USA 87, 8955–8959.
[11] Shimono, K., Iwamoto, M., Sumi, M. and Kamo, N. (1997) FEBS Lett. 420, 54–56.
[12] Klostermeier, D., Seidel, R. and Reinstein, J. (1998) J. Mol. Biol. 279, 841–853.
[13] Eilers, M. (1997) PhD Thesis, Universität Dortmund.
[14] Seidel, R., Scharf, B., Gautel, M., Kleine, K., Oesterhelt, D. and Engelhard, M. (1995) Proc. Natl. Acad. Sci. USA 92, 3036–3040.
[15] Hufnagel, P., Schweiger, U., Eckerskorn, C. and Oesterhelt, D. (1996) Anal. Biochem. 243, 46–53.
[16] Chizhov, I., Chernavskii, D.S., Engelhard, M., Müller, K.H., Zubov, B.V. and Hess, B. (1996) Biophys. J. 71, 2329–2345.
[17] Hirel, P.H., Schmitter, J.M., Dessen, P., Fayat, G. and Blanquet, S. (1989) Proc. Natl. Acad. Sci. USA 86, 8247–8251.
[18] Müller, K.H. and Plesser, H. (1991) Eur. Biophys. J. 19, 231–241.
[19] Müller, K.H., Butt, L., Bamberg, E., Fendler, K., Hess, B., Siebert, F. and Engelhard, M. (1991) Eur. Biophys. J. 19, 241–252.