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Circular dichroism and cross-linking studies of bacteriorhodopsin mutants

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Summary. Circular dichroism (CD) spectroscopy was employed for native (wild type, WT) bacteriorhodopsin (bR) and several mutant derivatives: R134K, R134H, R82Q, S35C, L66C, and R134C/E194C. Comparative analysis of the CD spectra in visible range shows that only R134C/E194C exhibits biphasic CD, typical for native bR, the other mutants demonstrate CD spectra with significantly smaller or absent negative band. Since the biphasic CD is a feature of hexagonal lattice structure composed by bR trimers in the purple membrane, these mutants and WT were examined by cross-linking studies, which confirmed the same trend towards trimeric organization. Therefore, a single amino acid substitution may lead to drastically different CD spectra without disruption of bR trimeric organization. Thus, although disruption of bR trimeric crystalline lattice structure (e.g., solubilization with detergents) directly results in the disappearance of characteristic bilobe in visible CD, the lack of the bilobe in the CD alone does not predict the absence of trimers.

Keywords: Bacteriorhodopsin – Mutants – Circular dichroism – Cross-linking – Matrix-assisted laser desorption/ionization mass spectrometry

Introduction

Bacteriorhodopsin (bR) is the sole protein of the purple membrane (PM) of *Halobacterium salinarium*, which functions as a light-driven proton pump. The chromophore of bR is retinal covalently bound via a protonated Schiff base to the ε-amino group of Lys-216. The PM structure is highly ordered, rigid, and immobilized (Cherry et al., 1977; Korenstein and Hess, 1978; Stoeckenius et al., 1982). BR molecules in the PM are arranged in trimeric clusters with P3 symmetry; trimers in turn form a two-dimensional hexagonal crystal (Henderson and Unwin, 1975; Henderson et al., 1990). Though at present bR is one of the best studied membrane proteins (see reviews: Haupts et al., 1999; Lanyi, 2004; Edmonds and Lucke, 2004), its structure–function relationship is still under intensive investigation using various bR analogs with synthetically modified retinals (Nakanishi and Crouch, 1995, review) and bR mutants (e.g., Tittor et al., 2002).

Circular dichroism (CD) spectroscopy, as a method sensitive to structural changes in the chiral system, has been extensively used to study bR structure including the protein arrangement in the membrane (Hasselbacher et al., 1988; Heyn, 1989) and bR conformational changes (Vogel and Gartner, 1987; Gibson and Cassim, 1989), as well as to characterize intermediates of the bR photocycle (e.g., Steinmuller et al., 1995), and heterogeneity and stability of bR in the PM (Friedman et al., 2003; Yokoyama et al., 2004; Sasaki et al., 2005). Retinal itself is an optically inactive molecule and thus does not exhibit any Cotton effect. Upon binding to apoprotein, retinal adopts a twisted (non-planar) conformation (Becher and Cassim, 1977; Haas and Buß, 1991; Volkov et al., 1997) and acquires chirality due to asymmetric protein environment that results in characteristic biphasic CD. The origin of the protein-induced chirality of retinal chromophore in bR has been a subject of numerous studies. Whereas absorption spectrum of native bR in the PM in visible range has a single broad band at 568 nm (light-adapted form), the corresponding CD spectrum is asymmetric bilobe with a positive band at 535 nm and a weaker negative band at 592 nm (non-conservative spectrum) with a zero crossover around 574 nm. The biphasic CD of bR was explained as a superposition of an intrinsic positive CD band caused by the protein environment and a conservative exciton couplet due to chromophore–chromophore interactions in the bR trimer (Heyn et al., 1975; Becher and Ebrey, 1976; Ebrey et al., 1977). To

date there is no perfect explanation on the bR biphasic CD band: possibilities include excitonic interaction between the chromophores in bR trimers (Becher and Cassim, 1977; Muccio and Cassim, 1979; Heyn, 1989), multiple transitions (El-Sayed, 1989; Birge and Zhang, 1990), and protein heterogeneity (Cassim, 1992; Wu and El-Sayed, 1991). However, there is general agreement that the biphasic CD band is observed exclusively for bR regular trimeric organization in the PM and the negative CD band at about 600 nm usually is considered as an indicator of the trimeric lattice structure (Casadio and Stoeckenius, 1980; Lam and Parker, 1983; Massotte and Aghion, 1991; Krebs et al., 1997). When PM is solubilized or is not completely regenerated (or partially bleached), the CD band becomes monophasic (Massotte and Aghion, 1991; Wu et al., 1991).

A single amino acid mutation has been shown to lead to significant changes in the bR CD spectrum (Jang et al., 1990; Krebs et al., 1997). Since the biphasic shape of the bR visible CD spectrum was often used as characteristic feature of trimeric aggregation and crystalline lattice structure, the altered CD spectra of the mutants were considered as an evidence of disruption of the bR lattice structure. It was shown (Krebs et al., 1997) that a single amino acid substitution in α-helix D was sufficient to disrupt the PM crystalline lattice. On the other hand, for the ''blue'' mutant D85N (absorption maximum at 605 nm) a monophasic CD band at 575 nm has been reported, despite the fact that trimeric crystalline structure was maintained (Kataoka et al., 1993). In the present work we demonstrate that a single amino acid substitution may, indeed, lead to an extremely different CD spectrum while bR trimeric organization is not necessarily disrupted. Here we show CD spectra of several bR mutants which differ from the original biphasic CD band typical for native bR, though the similarity of cross-linking enables us to predict a similar trimeric organization in the PM. (In designations of mutant pigments, the first letter represents the wild-type amino acid residue at the position indicated by the number and the second letter represents the substituted residue.)

We have employed conventional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and matrix-assisted laser desorption ionization mass spectrometry (MALDI MS) (Schey et al., 1992; Rosinke et al., 1995; Whitelegge et al., 1998) to examine the cross-linked membrane protein aggregates. We report here that the presence of the trimeric forms of these bR mutants does not correlate with the presence of a biphasic CD spectrum.

Materials and methods

Purple membrane

Control native PM suspension was obtained using wild type (S9) of *Halobacterium salinarium* by the conventional procedures (Oesterhelt and Stoeckenius, 1974; Becher and Cassim, 1975) and purified with a stepwise sucrose gradient (from 60% to 30%) at 23 000 rpm overnight. The mutants were prepared and characterized as previously reported (Lu et al., 2000) The membrane preparations of bR mutants R134K, R134H, R82Q, S35C, L66C, and R134C/E194C were purified similarly to those for bR. After sucrose removal all the samples had similar UV/Vis spectra with absorption maximum (λ_{max}) at 560 ± 2 nm. Purity criteria A_{280}/A_{562} OD ratio was 2.0–2.1. To provide relevant comparison of spectral measurements, all the bR stock preparations were adjusted to the same concentration (about 3.5×10^{-4} M).

Heterochromophoric sample bR/5,6-dihydro-bR (1:2) was prepared by stepwise regeneration of apo-membrane (Karnaukhova et al., 2006). Briefly, it was first regenerated with 0.33 molar equivalent of all-*trans* retinal, and this partially regenerated membrane sample (one positive CD band at 530 nm) was further coupled with 5,6-dihydro retinal (1.1 molar excess).

Chemicals

Formic acid, *N*-methylmorpholine, Triton X-100, protein internal standards bovine insulin, horse heart cytochrome *c*, horse skeletal muscle myoglobin, and bovine serum albumin were obtained from Sigma Chemical Co., St. Louis, MO. Dimethyl 3,3′-dithio bispropionimidate dihydrochloride (DTBP), dimethyl suberimidate dihydrochloride (DMS), dimethyl pimelimidate (DMP) were from Pierce, Rockford, IL. Hexafluoroisopropanol was from Brand-Nu Laboratories, Meriden, CT. Sinapinic acid was purchased from Aldrich, Milwaukee, WI.

UV/Vis and CD measurements

UV/Vis spectra were recorded on a Perkin-Elmer Lambda 6B UV/Vis spectrophotometer. CD measurements were performed on a JASCO J-700 spectropolarimeter using 1 cm quartz cuvette. UV/Vis and CD measurements were performed for all samples in a similar manner and the same conditions (in dark, at 25°C, with protein concentration of 3.5×10^{-4} M in 20 mM phosphate buffer).

Solubilization of the PM

PM was solubilized using Triton X-100 (final concentration of about 1% w/w) (Dencher and Heyn, 1982; Masotte and Aghion, 1991) in sodium phosphate buffer in the dark at room temperature for 30 h (with prior 30 s sonication on water bath). Solubilization of the PM was monitored by CD and UV/Vis. The solubilized sample was centrifuged at 30K for 15 min and the supernatant was used for the CD measurement. Solubilization accompanying sample preparation for SDS-PAGE and MALDI MS is described below.

Protein cross-linking

DTBP

The PM suspensions of WT and the mutants (2 mg) were spun down at 50K rpm for 15 min and the pellets were resuspended in 200 μl of 0.2 M *N*-methylmorpholine, pH 8.8. 18 mg of DTBP was dissolved in a mixture of 656 μl of 0.1 M *N*-methylmorpholine and 56 μl of ethanol. 100 μl of this solution was added to each bR sample, and the mixtures were stirred, sealed, and incubated at 37°C on a shaker for 2 h. After cross-linking, the

membrane samples were collected by centrifugation (50K, 15 min) and the pellets were washed 3 times with water.

DMS

The PM samples (2 mg) were spun down (50K rpm, 4°C, 15 min) and the pellets were resuspended in 0.5 ml of 0.1 M $NaHCO_3$. DMS (1 mg in 25 μl of distilled water) was added and the mixtures were incubated at 37°C on a shaker for 2 h. The samples were treated as described for DTBP.

DMP

The PM samples (2 mg) were pelleted and resuspended in 100 μl of 0.2 M triethylamine, pH 8.7. DMP (2.1 mg in 50 μl of 0.2 M triethylamine, pH 8.7) was added to each sample. The mixtures were treated as described for DTBP.

Gel electrophoresis

SDS-PAGE was performed using Novex Precast mini gel (12% Tris-Glycine). Proteins were visualized by staining with Coomassie BBR-250. The 10 kDa Protein Ladder (GibcoBRL) was used as a molecular weight standard.

MALDI-MS

Sample preparation

The samples were prepared according to Schey et al. (1992). Briefly, aliquots of the PM preparations (3 μl of 3.5×10^{-4} M stock suspension) were solubilized in 99% formic acid (7 μl) and 2 μl of hexafluoroisopropanol. After sonication for 1 min the solubilized membrane sample was centrifuged (30K for 5 min) and 1 μl of clear supernatant was mixed with 3 μl of 50 mM sinapinic acid in 70% formic acid. 0.5 μl aliquots of each resulting solution were loaded onto a gold-plated sample plate and allowed to air dry before measurements.

Mass spectrometer

A linear time-of-flight instrument with delayed extraction (Voyager-DE, Applied Biosystems, Framingham, MA) was used. To record mass spectra, usually 50–200 laser shots were averaged. Mass calibration was done using protein internal standards as equimolar mixture of bovine insulin (molecular weight, 5733.6), horse heart cytochrome *c* (12360.1), horse skeletal muscle myoglobin (16951.5), and bovine serum albumin (66430).

Results and discussion

Circular dichroism study

The CD spectra of native PM and the PM solubilized with Triton X-100 are shown in Fig. 1a. The biphasic form is lost on solubilization as previously reported (Heyn et al., 1975; Becher and Ebrey, 1976).

The CD spectra of five mutants of bR were measured: R134K, R82Q, S35C, L66C and R134C/E194C (Fig. 1b). Interestingly, only the double mutant, R134C/E194C exhibits the biphasic CD spectrum (Fig. 1b, trace 5), characteristic of native bR with a negative band at 593 nm and a positive band at 526 nm, similar to the CD spectra for native PM (trace 1, Fig. 1a) with cross-over corresponding to the pigment absorption maximum. The CD spectra of the other mutants do not have the characteristic biphasic shape. L66C shows a weak negative band (Fig. 1b, trace 4), while the negative band of S35C (trace 3) is insignificant and shifted to a longer wavelength (618 nm). The CD spectra of R134K, R82Q and R134H (not shown) are similar and all exhibit only a strong positive band around 540 nm. The monophasic shape of these spectra is closer to CD spectra of the solubilized PM (trace 2, Fig. 1a) than to the classical CD of the membrane bR (trace 1, Fig. 1a). For the PM, the shape of the non-conservative CD spectrum depends on the level of binding sites occupied by chromophore: partially bleached PM or not fully regenerated PM also exhibit mainly monophasic CD spectra. The intensity of the negative CD band increases when the percentage of regenerated PM exceeds

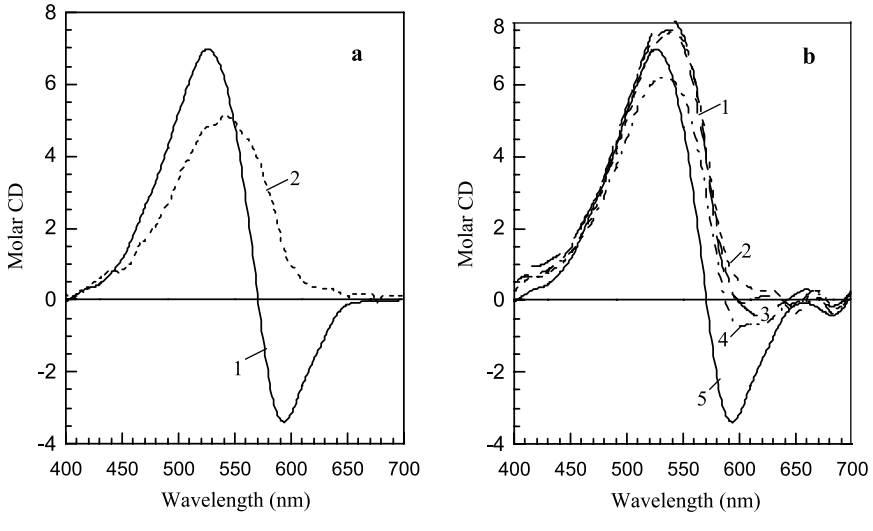

Fig. 1. CD spectra. **a** Native bR in the PM measured in 20 mM phosphate buffer (*1*) and PM solubilized with Triton X-100 (0.1%) in the same buffer (*2*); **b** bR mutants in 20 mM phosphate buffer: R134K (*1*), R82Q (*2*), S35C (*3*), L66C (*4*), and double mutant R134C/E194C (*5*). For all the mutants and native bR, the λ_{max} of the absorbance was in visible range at 560 ± 2 nm

45–46% (Wu and El-Sayed, 1991), which is consistent with growing occupancy of the chromophore binding sites with retinal and conformational changes leading to steric complementarity and crystalline lattice formation, and therefore is considered as a sign of a regular lattice structure. Thus, CD spectra of the mutants shown in Fig. 1b, aside from the double mutant, suggest the existence of non-aggregated bR. This observation prompted us to verify the similarity of the bR aggregation into trimers in the membrane.

Cross-linking

Intermolecular cross-linking was accomplished via amidination (Dellweg and Sumper, 1978; Baumert and Fasold, 1989) using homobifunctional imidoesters DTBP, DMS and DMP with spacer lengths of 11.9 Å, 11 Å and 9.2 Å, respectively (given according to Pierce product description information). At mild alkaline pH (7–10), imidoesters are known to react only with primary amines to form imidoamides. Figure 2 shows SDS-PAGE of PM cross-linked with DTBP. DMP gave similar results, but with a higher content of dimers in the mixture, while cross-linking with DMS was less efficient.

SDS-PAGE (Fig. 2) clearly showed a similarity of cross-linking for native bR and the mutants. Due to ease of aggregation of the PM particles in suspension, the efficiency of cross-linking was not high (Sigrist and Zahler, 1980; Baumert and Fasold, 1989), but it was found that the recovery of trimers could be much higher, if the procedure is repeated 2–3 times. Apparent molecular masses of bR monomer (22K), dimer (44K) and trimer (66K) typical for this sample preparation procedure (Sigrist and Zahler, 1980; Brouilette et al., 1987) were detected, but no traces of tetramer or polymer were found.

The heterochromophoric bR/5,6-dihydro-bR (1:2) (Karnaukhova et al., 2006) served as a negative control demonstrating a very faint band corresponding to cross-linked trimer (Fig. 2, lane 5). This faint band is likely caused by traces of initial non-bleached bR in apomembrane sample before regeneration or by traces of newly formed bR trimers during partial regeneration with all-*trans* retinal (the corresponding CD was monophasic). A splitting of the monomer band was observed for this sample on SDS-PAGE, though the mass difference between bR and 5,6-dihydro-bR subunits is negligible. This negative result on cross-linking for the heterochromophoric sample is important because it does not compose lattice structure due to *cis*-5,6-dihydro-bR (Spudich et al., 1986). The CD spectrum for this sample is additive (two positive bands at 462 nm and 530 nm directly corresponding to absorption spectrum).

MALDI MS of cross-linked bacteriorhodopsin

Figure 3 shows a typical MALDI mass spectrum of WT bR, similar to spectra previously reported (Schey et al., 1992). A singly protonated molecular ion $[M+H]^+$ at m/z 27 050 (predicted at m/z 27 068) is the dominant

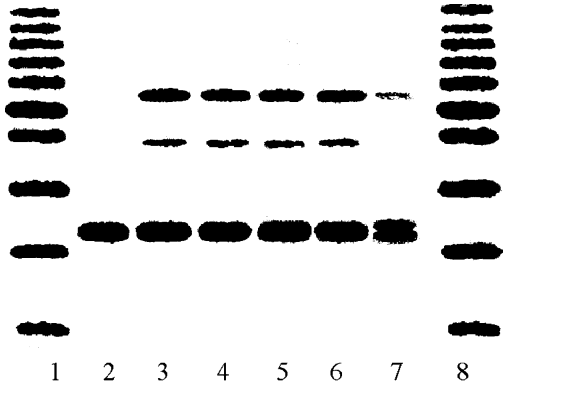

Fig. 2. SDS-PAGE of native PM (*2*), cross-linked PM (*3*) and cross-linked membrane preparations of mutants R82Q (*4*), R134K (*5*), R134C/E194C (*6*), and bR/5,6-dihydro-bR (1:2) sample after cross-linking (*7*). *1* and *8*, molecular weight standards

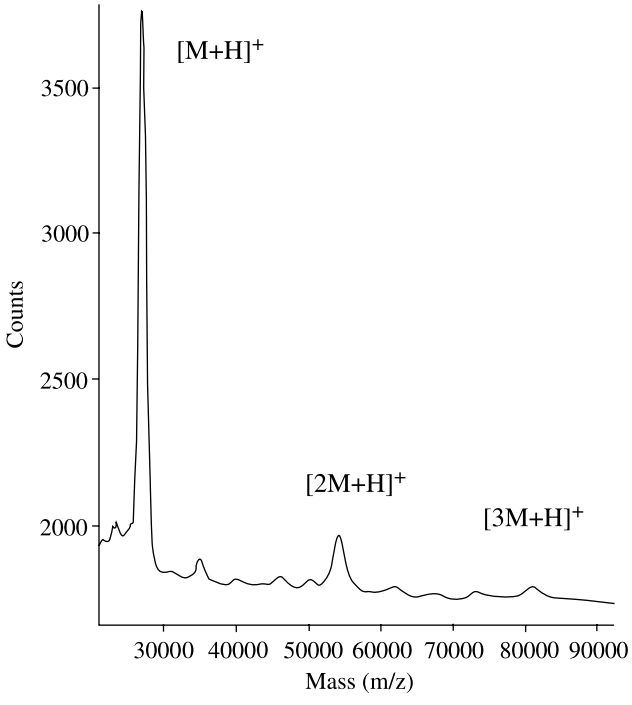

Fig. 3. MALDI mass spectrum of native WT PM before cross-linking

signal. The doubly charged monomer, $[M + 2H]^{2+}$ was detected at m/z 13 500 (data not shown). The singly charged dimer, $[2M + H]^{+}$, approximately m/z 54 100, is detectable. A small peak of singly charged trimer, $[3M + H]^{+}$, was identified at m/z 82 150. MALDI MS spectra of cross-linked samples (Fig. 4a–c), measured under the same conditions, are shown for comparison of non-cross-linked sample (Fig. 3). Since the membrane protein mixture after cross-linking and solubilization consists of trimer and dimer (covalently coupled species) as well as the bR monomer, overlapping of the corresponding unmodified molecular ions and those corresponding to derivatized bR would be expected. Nevertheless, by comparing spectral intensities (Figs. 3 and 4) one may conclude that peak III (Fig. 4a–c) is mainly the molecular ion of cross-linked trimer, and the small peak at m/z

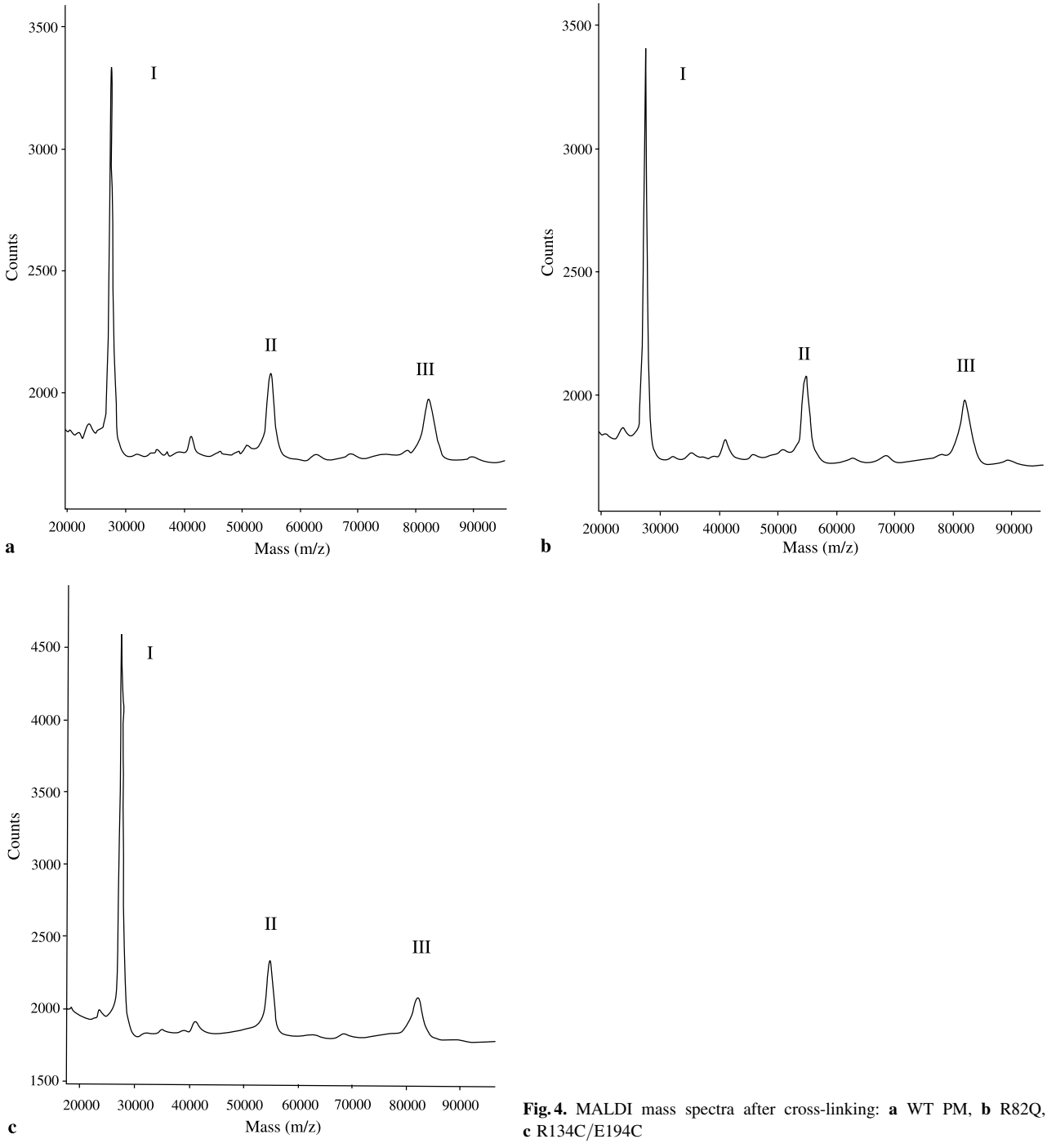

Fig. 4. MALDI mass spectra after cross-linking: **a** WT PM, **b** R82Q, **c** R134C/E194C

41076 is the doubly charged ion. The intensity of peak II results from the overlapping of the corresponding peaks from bR monomer and triply-charged trimer. The MALDI MS spectra of cross-linked samples (Fig. 4a–c) compared to control non-cross-linked samples (Fig. 3) confirm the identity of the cross-linked bR trimers as shown by SDS-PAGE (Fig. 2).

The measured mass of bR is in agreement with earlier published values (Schey et al., 1992; Whitelegge et al., 1998). The MALDI mass spectra of the cross-linked WT bR and the mutants (spectra of other mutants are similar to those shown in Fig. 4) indicate a very high similarity of protein trimeric aggregation, as confirmed by SDS-PAGE.

Conclusion

In a comparative analysis of CD spectra of WT bR and six mutants, significant differences were observed in the CD spectra. Only the CD spectrum of the double mutant R134C/E194C is close to classical biphasic CD typical for native bR in the PM. Cross-linking experiments undertaken for WT bR and bR mutants to examine the state of protein aggregation in their respective PMs showed the same trend toward trimeric aggregation. The observation that bR mutants with very similar trimeric organization in the PM may exhibit drastically different CD spectra means that the absence of biphasic CD does not necessarily indicate that bR trimeric organization is disrupted. This observation is evidence suggesting that the model used to explain the origin of CD of bR (Becher and Cassim, 1977; Muccio and Cassim, 1979; Heyn, 1989) requires further investigation.

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