Methionine Changes in Bacteriorhodopsin Detected by FTIR and Cell-Free Selenomethionine Substitution

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ABSTRACT Bacteriorhodopsin (BR) is an integral membrane protein, which functions as a light-driven proton pump in *Halobacterium salinarum*. We report evidence that one or more methionine residues undergo a structural change during the BR→M portion of the BR photocycle. Selenomethionine was incorporated into BR using a cell-free protein translation system containing an amino acid mixture with selenomethionine substituted for methionine. BR→M FTIR difference spectra recorded for unlabeled and selenomethionine-labeled cell-free expressed BR closely resemble the spectra of in vivo expressed BR. However, reproducible changes occur in two regions near 1284 and 900 cm^{-1} due to selenomethionine incorporation. Isotope labeled tyrosine was also co-incorporated with selenomethionine in order to confirm these assignments. Based on recent x-ray crystallographic studies, likely methionines which give rise to the FTIR difference bands are Met-118 and Met-145, which are located inside the retinal binding pocket and in a position to constrain the motion of retinal during photoisomerization. The assignment of methionine bands in the FTIR difference spectrum of BR provides a means to study methionine-chromophore interaction under physiological conditions. More generally, combining cell-free incorporations of selenomethionine into proteins with FTIR difference spectroscopy provides a useful method for investigating the role of methionines in protein structure and function.

INTRODUCTION

Fourier transform infrared *difference* spectroscopy is increasingly used to obtain information about protein conformational changes and has been applied recently to green fluorescent protein (van Thor et al., 1998), Ca^{2+}-ATPase (Barth and Mantele, 1998), phytochrome (Foerstendorf et al., 2001) and photosystem I from cyanobacteria (Kim et al., 2001). This method is especially applicable to the light activated archaeal rhodopsins found in *Halobacterium salinarum* including bacteriorhodopsin (BR), sensory rhodopsin I and II (SRI and SRII) and halorhodopsin (HR) (Bagley et al., 1982; Bergo et al., 2000; Bousche et al., 1991; Engelhard et al., 1996; Rothschild et al., 1988; Rothschild et al., 1981; Siebert and Maentele, 1983; Walter and Braiman, 1992).

In the case of BR, a variety of bands have been assigned in FTIR difference spectra to particular amino acids, including tyrosine, threonine, aspartic acid and tryptophan residues, which undergo a structural change during the BR photocycle. Examples of assignments to specific residues include Asp-85, the Schiff base counterion/proton acceptor (Braiman et al., 1988a) and Tyr-185 (Braiman et al., 1988b), located inside the retinal binding pocket. However, most amino acids residues have not yet been assigned to bands in these spectra, even though there exists strong evidence from mutagenesis studies and x-ray crystallography that many additional residues are involved in the BR photocycle. Assignment of these bands is important because it would provide a sensitive probe of BR structural changes during each step of the photocycle, thus complementing studies using x-ray crystallography. More generally, methods to assign bands in FTIR difference spectra would impact understanding of the detailed mechanism of a wide range of proteins amenable to this spectroscopic approach.

In this work, we have focused on identification of bands in the FTIR difference spectrum arising from methionine residues which undergo a structural change during the BR photocycle. Early studies of the role of methionine residues in the BR revealed the importance of Met-118 and Met-145 for proper protein function (Greenhalgh et al., 1993). More recently, comparison of the high-resolution structures of light-adapted BR (BR_{570}) and the M intermediate indicated that only these two methionines out of the nine present in the BR sequence undergo significant conformational changes during the BR→M transition (Luecke et al., 1999a). It is therefore likely that bands are present in the BR to M FTIR difference spectrum which reflect the changes in the structure and/or environment of these residues. Such an assignment could be valuable for studying the retinal interaction with these methionines under physiological conditions using time resolved infrared methods. The assignment of methionines vibrations in bacteriorhodopsin would also help facilitate similar studies in other proteins.

A second goal of this work is to evaluate a new approach for rapid labeling of specific amino acids or combinations of amino acids in a protein for the purpose of spectroscopic studies. Conventional labeling of proteins normally involves in vivo expression in the presence of a synthetic medium containing an isotopically labeled or analog amino acid accepted by the protein translational system. However, such an approach is slow and often requires auxotrophs that cannot synthesize the unlabeled form of the amino acid. The

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in vivo approach is particularly difficult if more than one amino acid needs to be labeled for the purpose of spectral editing, e.g., shifting interfering bands out of a particular region of the spectrum in order to confirm the presence of other bands in the region.

We have utilized here an alternative approach based on cell-free (e.g., in vitro) protein synthesis. Previously, cell-free protein synthesis was used along with a suppressor tRNA for the purpose of site-directed isotope labeling (SDIL) of tyrosines in BR (Sonar et al., 1994). For the purpose of assigning methionine bands, selenomethionine was partially substituted for methionine in bacteriorhodopsin by using a eukaryotic cell-free translation system. Previously, this approach was utilized for determination of the crystallographic structure of the RAS protein by multi-wavelength anomalous diffraction (MAD) phasing (Kigawa et al., 2001). Selenomethionine substituted bacteriorhodopsin samples (SeMet-BR) can be rapidly expressed, isolated and reconstituted. Two bands in the BR→M difference spectrum were found to undergo frequency shifts as a result of the selenomethionine substitution. The simultaneous incorporation of selenomethionine and L-tyrosine-[ring]-d_4 into BR was used in order to confirm the assignment of one methionine band. The cell-free labeling approach and particularly selenomethionine to methionine substitution should be generally applicable to a wide range of proteins for the purpose of FTIR band assignment.

MATERIALS AND METHODS

In vitro expression, affinity purification and refolding of selenomethionine labeled bacterioopsin

The synthetic bacterioopsin gene (*bop*) (Mogi et al., 1987) was expressed using the plasmid pβH6-bOp which is derivative of pUC19 (USB, Cleveland, OH) (Messing, 1983) (see Fig. 1). The plasmid was created from a construct kindly provided by Dr. Richard Timmer containing the T7 promoter, a 57 bp 5′ β-globin UTR (ACTTGCTTTTGACACAACT GTGTTTACTTGCAATCCCCCAAAACAGACAGATAGCTT) and the *bop* gene. This region was removed by *Hin*dIII digestion and ligated to *Hin*dIII digested pUC19 DNA. The resulting plasmid was digested by NotI-SacI and the DNA was ligated with synthetic DNA duplex coding for hexahistidine (CATCATCACCATCACCAT), which was incorporated immediately after the last codon in the *bop* sequence (TCT, Ser248) and prior to the two stop codons (TGA, TAA). Successful introduction of the His_6-tag was verified by dideoxy sequencing. The tag was introduced at the C terminus for convenient isolation of bacterioopsin (bOp) using Co^{2+} affinity column chromatography. The resulting plasmids was digested by *Hin*dIII and then transcribed with T7 polymerase using a commercial kit (mMESSAGEmMACHINE, Ambion, Inc., Austin, TX). Capped mRNA was purified by LiCl precipitation followed by ethanol precipitation.

Cell-free synthesis of wild type BR and selenomethionine BR analogs was carried out in a 6 ml volume of an mRNA-dependent wheat germ translation mixture (Promega Corp., Madison, WI) to which 10 μCi of ^{14}C-Leu (Amersham, Cleveland, OH) and 50 μg/ml of mRNA was added. Incorporation of selenomethionine into bOp during translation was optimized using an amino acid mixture (100 μM) lacking methionine and supplemented with varying amounts of selenomethionine (see Results). A similar procedure was also used in order to incorporate L-tyrosine-[ring]-d_4 into bOp (both selenomethionine and L-tyrosine-[ring]-d_4 were used at 2 mM concentration). Synthesis was performed at 27°C for 2 h. The product of cell-free synthesis was analyzed by SDS-PAGE (12%) followed by fixing, drying and autoradiography using a phosphorimager system (Storm 860, Molecular Dynamics).

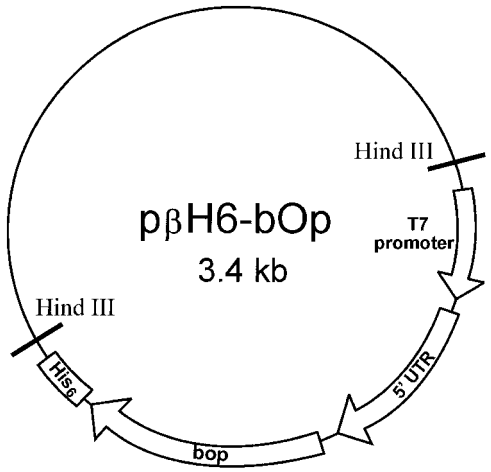

FIGURE 1 pβH6-bOp plasmid which is a derivative of the pUC19 plasmid (see Materials and Methods). See supplemental material for sequence of the T7 promoter, 5′ UTR, *bop-His$_6$* region.

Purification of in vitro produced bOp was carried out using Co^{2+} affinity purification resin (Talon, Clontech). 2 ml of PEG-8000, followed by 500 μl of 10% SDS and 500 μl of 1 M Tris-HCl at pH 8.0 were added to 6 ml of wheat germ translation mixture. To this solution 2 ml of the Talon resin equilibrated with 100 mM Tris-HCl, pH 8.0 was added and the suspension incubated for 20 min with gentle shaking. This mixture was then loaded on a 1 cm × 10 cm disposable column and after the resin settled washed 5 times with 1 ml of wash buffer (100 mM Tris-HCl, pH 8.0; 1% SDS). Bound bOp-His_6 was eluted from the column by washing with elution buffer (wash buffer containing 200 mM imidazole). After addition of the elution buffer, 550 μl fractions were collected and 10 μl aliquots analyzed by SDS-PAGE (12%), Coomassie staining and scintillation counting.

Refolding of in vitro expressed bacteriorhodopsin

Refolding of in vitro expressed BR was based on a previously described method (Popot et al., 1987). All-*trans* retinal, sodium taurocholate, and buffer salts were purchased from Sigma (St. Louis, MO). Polar halobacterial lipids were prepared as described before (Popot et al., 1987). Pooled fractions of affinity purified bOp (0.8–1.0 ml) were dialyzed overnight against 500 ml of 0.5% (w/v) SDS, 50 mM NaP_i (pH 6.0) using disposable Filtrasep dialysis tubes with MW cutoff of 8,000 (Midwest Scientific, St. Louis, MO). The sample was then freeze-dried, redissolved in 100 μl of distilled water and dialyzed again as described above (two buffer changes total). After dialysis to the bOp solution were added (250–300 μl final volume): 7 μl of 1% (w/v) sodium taurocholate solution in 5% (w/v) SDS, 50 mM NaP_i, pH = 6.0; 20 μl of 0.5% w/v halobacterial lipids solution in 5% SDS; 50 mM NaPi pH = 6.0; and 2 μl of all-*trans* retinal solution (5 mM in ethanol). The resulting solution was vortexed briefly, incubated at room temperature for 15 min and then 40 μl of 4M KCl solution was slowly added. The resulting slurry was vortexed briefly and the tube was repeatedly inverted during the next 10 min. The sample was then incubated in darkness overnight at room temperature. The next day the suspension was vortexed briefly and then centrifuged at 500 rpm for ~30 s. The supernatant was

collected and transferred to a new tube. The white precipitate was washed with 50 μl of dH_2O, centrifuged, and the supernatant combined with the first portion. The resulting solution was then dialyzed for 4–5 days against 500 ml of 150 mM KCl, 50 mM KP_i (pH = 6.0), the buffer was changed daily. The resulting solution was freeze-dried, resuspended in 100 μl of dH_2O, centrifuged at 16,000 rpm for 20 min in tabletop centrifuge (Eppendorf model 5415C) and supernatant discarded. The pellet of refolded protein was resuspended in 50 μl of dH_2O, vortexed, and centrifuged again (2 times).

FTIR Difference Spectroscopy

Films were prepared by depositing 10 μl of the wet pellet of the refolded in vitro expressed BR onto an AgCl window and then placing the sample in a dry-box for ~1 h. Films were then rehydrated by placing 1–1.5 μl of H_2O near the edge of the window and sealing the sample in a temperature-controlled IR cell (Model TFC, Harrick Scientific Corp., Ossining, NY) using a second AgCl window. Spectra were recorded as previously reported at −20°C and 2 cm^{-1} resolution (Roepe et al., 1987) with a Bio-Rad FTS-60A FTIR spectrometer (Bio-Rad, Digilab Division, Cambridge, MA) using a liquid nitrogen cooled MCT detector. A Dolan-Jenner (Woburn, MA) model 180 illuminator (150 W, tungsten-halogen) and a fiber-optic light guide were used for sample illumination in combination with a long pass $\lambda_{max} > 505$ nm filter (Corion Corp., Holliston, MA). Spectra were typically recorded of the sample in the dark and light for 1400 scans each and the absorption difference computed. At least 20 absorption difference spectra are recorded and averaged to obtain the final difference spectrum.

RESULTS AND DISCUSSION

Selenomethionine incorporation into in vitro expressed bacterioopsin

To optimize selenomethionine incorporation into the in vitro produced bacterioopsin, measurements were made to determine the effect of adding different concentration of selenomethionine or methionine on the translation yield. Small scale translation reactions (50 μL) were carried out using wheat germ extract, capped bOp mRNA, ^{14}C-Leu, and amino acid mixtures containing varying concentrations of methionine or selenomethionine. The amount of bOp produced was then determined by using TCA precipitation combined with scintillation counting or by SDS/PAGE combined with phosphorimager detection (Promega, 1996).

As seen in Fig. 2, bOp is produced in the wheat germ extract, even in the absence of exogenously added methionine. This indicates that the wheat germ extract contains methionine either in a free form or as Met-$tRNA^{Met}$. The synthesis of bOp increases ~twofold above this basal level with increasing concentrations of exogenous methionine up to 100–200 μM, with a slight decrease observed at higher concentrations (Fig. 2). Note that at the highest concentration (2000 μM) (Fig. 2, *inset*) substitution of selenomethionine for methionine in the reaction mixture had minimal effect on the yield of bOp. This agrees well with previously published results on selenomethionine substitution in a cell-free produced Ras (Kigawa et al., 2001). On this basis, we conclude that selenomethionine is accepted almost as well as methionine by the in vitro translation system. Since the overall yield of bOp is twofold higher above basal

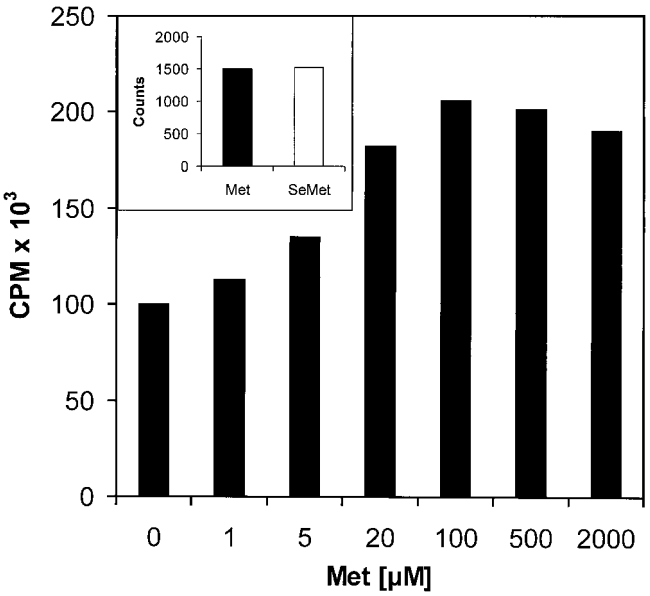

FIGURE 2 bOp production vs. exogenous methionine concentration. A series of translation experiments were performed with increasing concentration of methionine. An aliquot was withdrawn after 120 min and precipitated using TCA, followed by measurement of radioactivity using scintillation counting. Inset: Yield of translation product at 2 mM concentration of methionine and selenomethionine as analyzed by SDS-PAGE followed by detecting using a phosphorimager.

level when SeMet is added, the SeMet→Met substitution should be at least 50%. However, SeMet incorporation is likely to be higher since the endogenous methionine which accounts for protein synthesis when no external methionine (or selenomethionine) is added, will be diluted by the selenomethionine added to the reaction mixture. Importantly, as discussed below, this level of incorporation is sufficient to detect alterations in the FTIR difference spectra.

Preparative samples of SeMet-BR were prepared by performing in vitro expression in a total volume of 6 ml of the wheat germ extract and with a 2 mM concentration of selenomethionine. Purification was achieved on a Co^{2+} affinity column using the incorporated C-terminal His_6-tag. The purity of the protein sample was then compared with that of in vivo expressed BR using SDS-PAGE (Fig. 3). It can be seen that the in vivo expressed BR appears as expected as a single band at ~26 kDa. Similarly, a single band corresponding to SeMet-BR appears at a slightly higher position due to the presence of the additional His_6-tag.

FTIR difference spectroscopy

Fig. 4 shows the BR→M difference spectra of in vitro expressed BR (WT) (*bottom trace*), and the selenomethionine containing in vitro expressed BR (SeMet-BR) (*top trace*) in two regions of the spectrum from 1800–1400 cm^{-1} (panel *A*) and 1400–1000 cm^{-1} (panel *B*). Previous studies of BR have shown that FTIR difference spectroscopy is

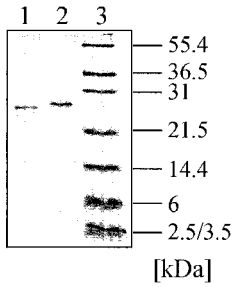

FIGURE 3 SDS-PAGE/Coomassie stain of in vivo produced WT bR (*lane 1*) and in vitro produced and purified SeMet-BR (*lane 2*). Lane 3 contains size markers (Mark12, wide range standard, InVitrogen, Carlsbad, CA).

highly sensitive to the conformational changes that occur during the BR→M transition (Rothschild and Sonar, 1995). Even small perturbations in the pattern of hydrogen bonding changes, which occur on a local level (e.g., individual side chains), should be detectable in the difference spectrum.

In the case of SeMet-BR, the very similar appearance of its FTIR difference spectrum compared to WT BR indicates that the Met→SeMet substitution has very little effect on the normal structural changes occurring in the BR photocycle. For example, no effects are seen in the carboxylic acid (C═O) Asp/Glu stretching region (1800–1700 cm^{-1}), amide I region (1700–1600 cm^{-1}) (Parker, 1983), the ethylenic C═C stretch region of the retinylidene chromophore reflecting the shift from light adapted BR (1527 cm^{-1} (−)) to the M intermediate (1567 cm^{-1} (+)) and the chromophore fingerprint region (1254 (−), 1201 (−), 1168 (−) cm^{-1}) (Smith et al., 1987). Bands assigned to particular amino acids are equally unaffected. Examples include peaks due to Asp-85 at 1761 cm^{-1} (+), Asp-96 at 1742 cm^{-1} (−) (Braiman et al., 1988a; Maeda et al., 1992b), Tyr-185 at 1276 cm^{-1} (−) and 1271 cm^{-1} (+) (Braiman et al., 1988b; Roepe et al., 1987; Rothschild et al., 1986), Thr-89 near 1125 cm^{-1} (Liu et al., 1998); and Trp in the 740 cm^{-1} region (Roepe et al., 1988) and at 3486 cm^{-1} (Maeda et al., 1992a) (data not shown). The vibrational bands assigned to at least one water molecule, most likely located in the active site of BR (Brown et al., 1994), are also not altered by the SeMet substitution (data not shown).

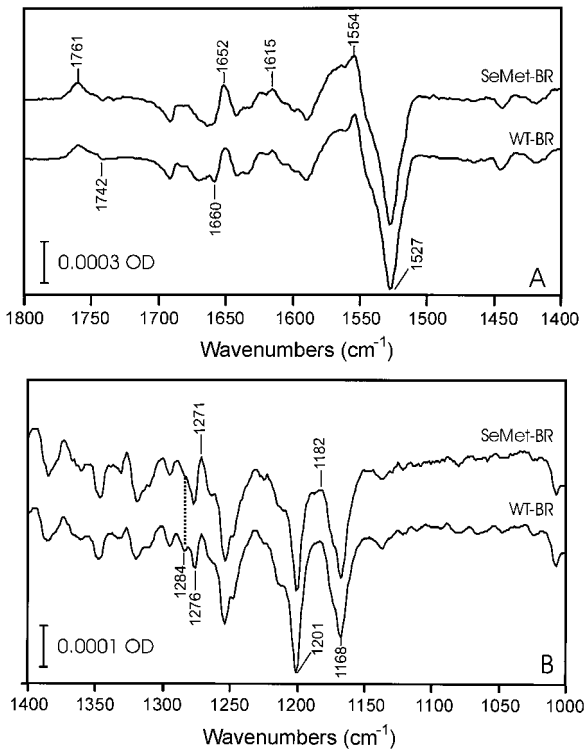

FIGURE 4 FTIR difference spectra of in vitro expressed wild type BR (WT-BR) and selenomethionine substituted BR (SeMet-BR) recorded at 2 cm^{-1} resolution and −20°C. Top trace: SeMet-BR. Bottom trace: WT-BR in the 1400–1800 cm^{-1} region (*panel A*) and 1000–1400 cm^{-1} region (*panel B*). See Materials and Methods for additional information.

Despite the similarity between the FTIR difference spectra of SeMet-BR and WT BR, we observe a reproducible change of a negative band appearing at 1284 cm^{-1} in WT BR which is absent in SeMet-BR (Fig. 4 *B*). This region has been previously associated with structural changes in the protonation state of tyrosine. In particular, the 1276 (−) and 1271 (+) cm^{-1} bands were assigned to alterations in the environment of a tyrosine on the basis of in vivo amino acid substitution of L-tyrosine-[ring]-d_4 for normal L-tyrosine (Roepe et al., 1987; Rothschild et al., 1986). Site-directed mutagenesis (Braiman et al., 1988b) and site-directed isotope labeling (Liu et al., 1995) further demonstrated that these bands arise from Tyr185.

To confirm that the spectral change at 1284 cm^{-1} was solely due to the Met→SeMet substitution, we spectrally edited this region to remove interference from tyrosine bands. This was accomplished by substituting Tyr with L-tyrosine-[ring]-d_4 (D_4-Tyr-BR) both for normal and SeMet substituted BR (see Materials and Methods). Spectral changes induced by L-tyrosine-[ring]-d_4 substitution (second trace from top, Fig. 5) agree closely with previous studies using in vivo substitution of tyrosine and include almost complete disappearance of the bands at 1276 cm^{-1} (−) and 1271 cm^{-1} (+) (Roepe et al., 1987). This result indicates that in vitro L-tyrosine-[ring]-d_4 substitution is highly efficient.

As seen in Fig. 5, the 1284 cm^{-1} negative band is still present in D_4-Tyr-BR indicating that it does not arise from tyrosines. However, the double substitution of L-tyrosine-[ring]-d_4 and SeMet (D_4-Tyr/SeMet-BR) (Fig. 5, *top trace*) clearly shows the disappearance of this band. We conclude on this basis that the 1284 cm^{-1} band arises from alteration of one or more Met during the BR →M portion of the photocycle. There is some evidence that this band *upshifts* in frequency to the 1310-1320 cm^{-1} region due to Met→SeMet substitution, which is indicated by a small downshift in frequency of a strong band located near 1320 cm^{-1} to near 1318 cm^{-1} for both the case of D_4-Tyr/SeMet-BR and SeMet-BR.

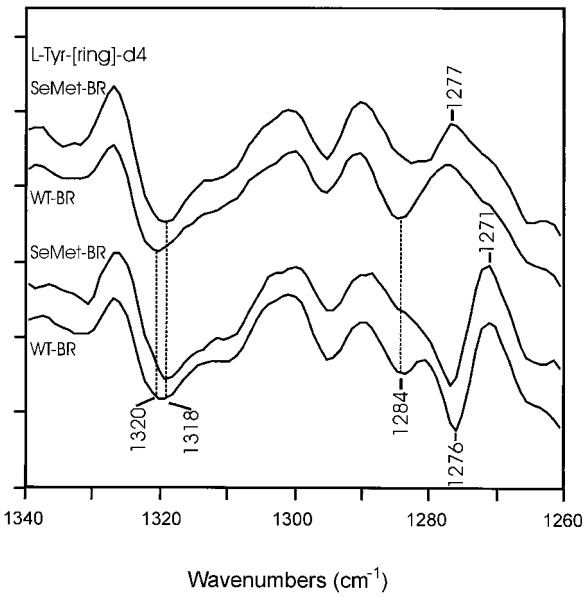

FIGURE 5 FTIR difference spectra of in vitro expressed WT-BR (*bottom trace*), SeMet-BR (*second from bottom*), L-tyrosine-[ring]-d_4 labeled BR (*second from top*), and SeMet-BR containing L-tyrosine-[ring]-d_4 label (*top trace*) in the region from 1260–1340 cm^{-1} recorded under same conditions specified in Fig. 4. Spacing of the *Y*-axis (absorbance) markers corresponds to 2×10^{-4} OD.

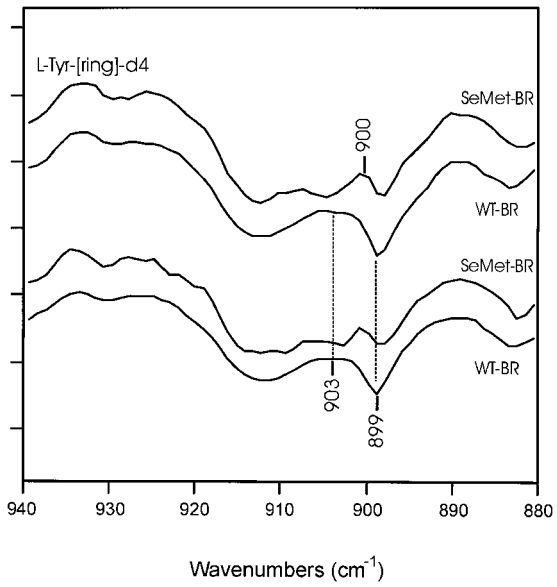

FIGURE 6 FTIR difference spectra of in vitro expressed WT-BR (*bottom trace*), SeMet-BR (*second from bottom*), L-tyrosine-[ring]-d_4 labeled BR (*second from top*), and SeMet-BR containing L-tyrosine-[ring]-d_4 label (*top trace*) in the region from 880–940 cm^{-1} recorded under same conditions specified in Fig. 4. Spacing of the *Y*-axis (absorbance) markers corresponds to 2×10^{-5} OD.

A second reproducible spectral change is observed in both BR and D_4-Tyr-BR at 899 cm^{-1} (−). This negative band disappears (Fig. 6) and is accompanied by a drop in intensity of a positive peak near 903 cm^{-1}. A possible explanation is that SeMet substitution causes the 899 cm^{-1} band to upshift 3–4 cm^{-1}. We also note that it is possible that upon M formation a shift in frequency occurs for both methionine assigned negative bands at 1284 and 899 cm^{-1} giving rise to positive bands elsewhere in the spectrum. However, since we have not identified such positive components, the most likely explanation is that both these bands undergo primarily a change in intensity.

Model compounds

Earlier infrared studies of methionine and selenomethionine, which utilized solid-state amino acids (Shepherd and Huber, 1969; Tamba et al., 1973), revealed that the S→Se substitution had a small effect on the 1800–1400 cm^{-1} region but altered many peaks below 1400 cm^{-1}. The latter bands represent various vibrational modes of the methionine side chain and typically have medium intensity (Grunenberg and Bougeard, 1986). Because our difference spectra exhibited only two bands at 1284 and 903/899 cm^{-1}, which are sensitive to the S→Se replacement, it appears that only minor alterations of the chemical bonds involving sulfur occur during the BR→M transition. While assignment of these bands to particular vibrational modes of methionine is not possible based on the current data, one infrared study of solid methionine (Grunenberg and Bougeard, 1987) observed bands at similar frequencies (1276 and 921 cm^{-1}) (intensities not given), which were assigned to predominantly S-C-H bending modes. Alternatively, the 1284 cm^{-1} peak may arise from the mixed CH_3-deformation and S-C-H bending vibration observed between 1311–1318 cm^{-1} (Grunenberg and Bougeard, 1987; Tamba et al., 1973). This band shifts to 1345 cm^{-1} upon substitution with selenium (Tamba et al., 1973), which would be consistent with the possible 36 cm^{-1} upshift of the 1284 cm^{-1} band.

CONCLUSIONS

FTIR difference spectroscopy can be used as an effective tool for exploring protein structural changes and functional mechanisms provided bands can be assigned to the vibrations of specific amino acids and ultimately to individual residues. In this study, we have utilized a new approach based on *cell-free* protein expression for assignment of bands to specific amino acids. This approach has several advantages over the in vivo incorporation of stable isotopes and amino acid analogs into proteins. First, since most biochemical pathways are not functional in the in vitro extract, scrambling of an isotope label is avoided. This allows a combination of isotopic amino acid labels and even amino acid analogs to be *simultaneously* incorporated into the protein. For example, we were able to incorporate both L-tyrosine-[ring]-d_4 and selenomethionine for the purpose of isotope editing of interfering bands from tyrosine vibrations.

A second advantage is the ability to rapidly express protein samples at levels sufficient for FTIR-difference spectroscopy. For example, in the case of BR, 6 ml of wheat germ extract produces ~10 μg of purified, refolded BR, sufficient to obtain high quality FTIR difference spectra. Utilization of dialysis methods of cell-free protein translation should produce even higher yields and higher levels of isotope/analog amino acid incorporation (Kigawa et al., 2001).

Our results indicate that one or more methionines undergo structural changes during the BR photocycle. An important question is the identity of those specific methionine residue(s), which give rise to the methionine assigned bands. Studies based on site-directed mutagenesis by Khorana and coworkers (Greenhalgh et al., 1993) revealed that two methionines, Met-118 and Met-145, are important for proper BR folding, λ_{max} and photocycle behavior of BR.

More recently, high-resolution x-ray crystallography has revealed the location of methionines that undergo structural changes during the BR photocycle (Luecke et al., 1999a; Luecke et al., 1999b). As was predicted by mutagenesis studies, Met-118 is located in the retinylidene binding site and interacts directly with the C9-methyl group of retinal, while Met-145 is located on helix E and near the retinal β-ionone ring of the chromophore. Analysis of the crystal structures of the light-adapted and M photointermediate states using protein data bank (PDB) coordinate files 1C8R and 1C8S (Luecke et al., 1999a), shows that only these two methionines experience a noticeable structural change during the BR→M transition. The largest changes occur in Met-118 where the side chain adopts a different conformation causing the displacement of the terminal methyl group by more than 2 Å from its ground state position. Residues near the β-ionone ring including Met-145 experience only minor alterations that result from a small (0.4 Å) movement of this part of the chromophore after isomerization (Luecke et al., 1999a). Therefore the appearance of vibrational features associated with methionine in the BR→M difference spectra could be explained by the changes in the conformation of methionine side chain(s) observed in the x-ray studies. Note that significant perturbations of methionine groups occur only in the late M (M_N) intermediate, which is presumably formed under conditions of our experiment (steady-state illumination at –20°C). In contrast the structure of the early M state 1KG8 (Facciotti et al., 2001) obtained at lower temperature (230 K) exhibited much smaller protein changes. Similarly, the structure of the early K intermediate (PDB 1QKP) did not show any changes of Met-118 or Met-145, although a small movement of Met-20 side chain in response to the chromophore isomerization was observed (Edman et al., 1999).

In conclusion, by using cell-free amino acid substitution, it should be possible to quickly assign bands in the FTIR difference spectrum of many proteins. For example, once a protein can be expressed and purified in a cell-free system, it is relatively simple to introduce a variety of amino acid mixtures containing single amino acid isotope labels or amino acid analogs such as selenomethionine. This may be especially valuable in the case of proteins that are normally expressed in insect cell cultures (Sf9) or mammalian cell cultures, where isotope labeling is more difficult to achieve.

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