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Localisation of methionine residues in bacteriorhodopsin by carbonyl ^{13}C-NMR with sequence-specific assignments

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High-resolution ^{13}C-NMR experiments have been performed on bacteriorhodopsin biosynthetically labeled with carbonyl-^{13}C amino acids and solubilized in the detergent dodecylmaltoside. ^{13}C-NMR spectra showing good resolution were obtained in the case of labeled amino acids moderately represented in the BR sequence. For BR labeled with [^{13}C]carbonyl methionine, several sequence-specific assignment could be performed by co-labeling with ^{15}N amino acids or proteolysis. These assignments were used to obtain structural data on BR. Water-exposure of methionine side chains in the protein was assessed by studying, using NMR, their oxidation by hydrogen peroxide. Local secondary structure at the level of methionine residues was monitored through the effect of ^{1}H–^{2}H exchange on NMR spectra. It was concluded that Met^{32}, Met^{68} and Met^{163} are peripheral while all 6 other methionine residues are deeply embedded within hydrophobic α-helices. These results confirm the current model of the BR folding and secondary structure.

Bacteriorhodopsin; NMR, Membrane protein structure; Biosynthetic labeling

1. INTRODUCTION

In recent years, important progress has been made towards the understanding of the three-dimensional structure of bacteriorhodopsin (BR), from *H. halobium*. This has been obtained from diffraction studies on two-dimensional BR crystals [1,2] or from site-directed mutagenesis experiments [3]. Other approaches have to be used in order to ascertain these results as well as to investigate the many aspects of the bacteriorhodopsin structure that remain unknown. In this instance, high-resolution NMR is a powerful technique for the structural study of proteins up to the 20 kDa m.wt. range (for review see [4]). We have recently attempted to apply this approach to detergent-solubilized BR. Our previous work has been devoted to design procedures for preparing detergent-solubilized BR samples yielding high-resolution and signal-to-noise ratio in NMR spectra [5] and to study the topography and dynamics of particular residues in the BR structure by ^{13}C-NMR [6].

Quantitative NMR studies of BR requires both the ability to resolve a large number of single-residue resonances and to perform sequence-specific assignments for these resonances. ^{13}C-NMR after biosynthetic labeling with carbonyl-labeled amino acids has been shown for several soluble proteins [7–10] and a small membrane protein [11,12] to provide adequate resolution and assignments. In the present study we evaluate the resolution attainable by such an approach with labeled amino acids variously represented in the BR sequence. We also report on sequence-specific assignments of several resonances in the particular case of BR containing ^{13}C carbonyl-labeled methionine. By monitoring the effect of extrinsic agents on NMR spectra, we also assess the localisation of the various methionine residues with regards to the external aqueous regions of BR. The results are used to evaluate the current folding and secondary structure model of BR.

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2. MATERIALS AND METHODS

Single ^{13}C labeling or ^{13}C–^{15}N double labeling of BR was effected by growing *H. halobium* (strain S9) using the medium described in [13] in which one or two of the unlabeled amino acids were replaced by the corresponding ^{13}C and ^{15}N amino acids. Purple membrane purification and proteolysis of BR were performed as described [14,15]. BR was delipidated and solubilized using our previously published procedure [5] at a final concentration of 17–19 mg/ml in 5% (w/v) dodecylmaltoside, 20 mM MES, pH 6. The solubilized BR was adjusted to pH 5 and diluted to 8.5 mg/ml for NMR experiments. All samples contained 5% (v/v) $^{2}H_2O$ except where otherwise stated. The singly and doubly labeled BR used in this study are refered to using the one letter code e.g. [1,^{13}C]methionine-labeled BR is termed (M)BR and [1,^{13}C]methionine and [^{15}N]glycine doubly labeled BR (M,G)BR.

^{1}H-decoupled ^{13}C-NMR spectra were recorded at 50°C in 10 mm tubes using a Varian XL300 spectrometer operating at 75.4 MHz. Parameters used for acquisition were a 90° pulse angle, a 16.5 kHz spectral width, a 0.5 s acquisition time and a 2.5 s recycle time. A MLEV16 sequence was used for ^{1}H decoupling. Chemical shifts are reported relative to external aqueous dioxane. Except where otherwise stated, spectra were processed with a 4 Hz line broadening. The extent

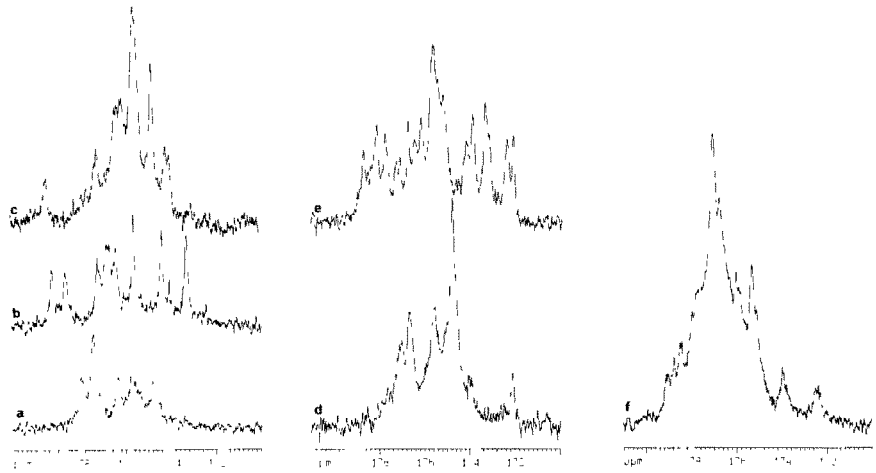

Fig. 1. ^{13}C-NMR spectra recorded at 50°C of BR (8 mg/ml) labeled with various (1,^{13}C) amino acids and solubilized in 2.5% (w/v) n-dodecylmaltoside. (a) (K)BR, (b) (M)BR, (c) (F)BR, (d) (I)BR, (e) (V)BR, (f) (L)BR

of BR denaturation was routinely checked by visible absorption spectrometry after NMR experiments and found to be less than 6% except when otherwise mentioned

3. RESULTS

3.1. *^{13}C NMR spectra of ^{13}C-carbonyl amino acid-labeled BR*

Our previous work indicated that BR solubilized in dodecylmaltoside is stable at temperatures up to 50°C and provides optimal resolution for solution NMR experiments [5]. To further investigate the attainable resolution, ^{13}C-NMR spectra of BR samples labeled with various carbonyl ^{13}C amino acids were recorded in dodecylmaltoside at 50°C. As judged from Fig. 1, the main factor influencing the resolution appears to be the number of residues of the corresponding amino acid in the BR sequence. For amino acids scarcely represented, such as lysine and methionine (respectively 7 and 9 residues per BR), almost all individual carbonyl resonances are resolved on the corresponding NMR spectra of (K)BR and (M)BR. In the case of (F)BR, (I)BR and V(BR), that correspond to intermediately represented amino acids (respectively 12 phenylalanine, 16 isoleucine and 21 valine residues per BR), partial resolution is obtained. On the other hand, for (L)BR, a relatively low resolution spectrum is obtained consistently with the occurrence of 36 leucine residues in BR. The resolution is also influenced by the chemical shift dispersion of the carbonyl ^{13}C resonances which, interestingly enough, depends on the labeled amino acid. This can be accounted for by the fact [16,17] that ^{13}C carbonyl resonances of residues inside α-helices tend to appear at lower field. In this instance, the observed chemical shift dispersion appears to be in agreement with the limits of transmembrane helices proposed by Henderson et al. [2]. There is apparently a good correlation between the proportion of the NMR intensity at high field (i.e. below 176–177 ppm) and percentage of residues outside transmembrane helices that these authors inferred (respectively 31, 29, 23, 20, 17, and 6% for Ile, Lys, Met, Val, Phe and Leu).

3.2. *Sequence-specific assignments in the ^{13}C-NMR spectrum of (M)BR*

The possibility of obtaining sequence-specific assignments in the ^{13}C-NMR spectra was investigated in the

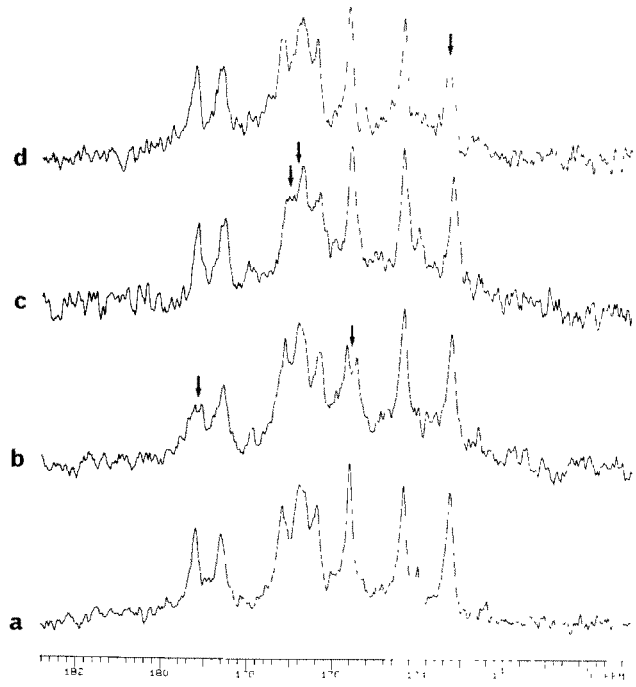

Fig. 2. ^{13}C-NMR spectra recorded at 50°C of BR (8 mg/ml) labeled with [1,^{13}C]methionine or doubly labeled with [1,^{13}C]methionine and with various ^{15}N amino acids and solubilized in 2.5% (w/v) n-dodecylmaltoside. (a) (M)BR, (b) (M,G)BR, (c) (M,L)BR, (d) (M,R)BR. The arrows indicate the positions of the ^{13}C resonances affected by ^{15}N labeling

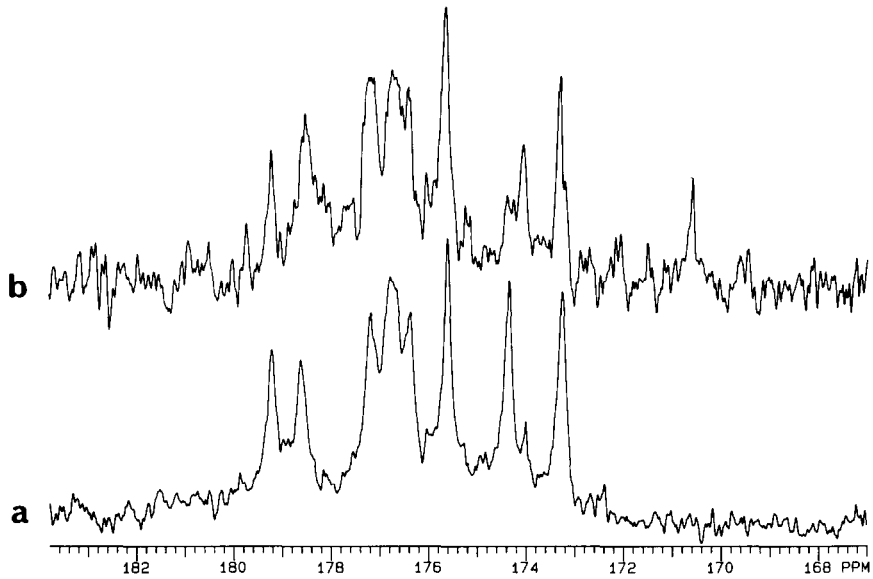

Fig. 3. ^{13}C-NMR spectra recorded at 50°C of (M)BR (8 mg/ml) intact (a) or pretreated with papain (b) and solubilized in 2.5% n-dodecylmaltoside.

case of (M)BR with which 7 methionine carbonyls over 9 give rise to single resolved lines, the remaining two yielding overlaping lines at 176.8 ppm (Fig. 2). One method used for assignment was ^{13}C–^{15}N double labeling which takes advantage of the splitting or broadening of the ^{13}C carbonyl resonance that occurs when the corresponding amino acid is directly linked to an ^{15}N-labeled amino acid [7,8]. ^{15}N amino acids corresponding to immediate neighbors of methionine residues in the BR sequence were thus incorporated in the protein together with ^{13}C carbonyl-labeled methionine. As in other cases [18] a difficulty in these experiments was the occurence of aminotransferase activities in *H. halobium* which scrambled the initially specific ^{15}N labeling. Thus, it was possible to observe the effect of the ^{15}N labeling on the ^{13}C-NMR spectrum only with 3 doubly labeled samples. The case of (M,G)BR was particularly favorable since very high ^{15}N incorporation presumably occured (glycine not being usually a substrate for aminotransferases, see [18]) leading to the splitting and the broadening of the ^{13}C resonances at 179.3 and 175.6 ppm, respectively. These are thus attributed to Met^{20} and Met^{32} (see below for the relief of this ambiguity). Similarly, the two broadened resonances at 177.2 and 176.8 ppm in the spectrum of (M,L)BR are attributed to Met^{60} and Met^{145}. In the case of (M,R)BR a limited (due to low ^{15}N incorporation) but significant broadening of the high-field 173.3 ppm resonance occurs, leading to its assignment to Met^{163}.

For the purpose of assigning the resonance of Met^{68}, the effect of papain proteolysis of BR on the ^{13}C-NMR spectrum was monitored. Such treatment cleaves the segment corresponding to residues 67–72 [15]. Unlike the intact protein, the proteolysed BR underwent progressive denaturation in dodecylmaltoside at 50°C. By halving the acquisition time it was possible to obtain the spectrum of Fig. 3b while keeping to 26% the extent of

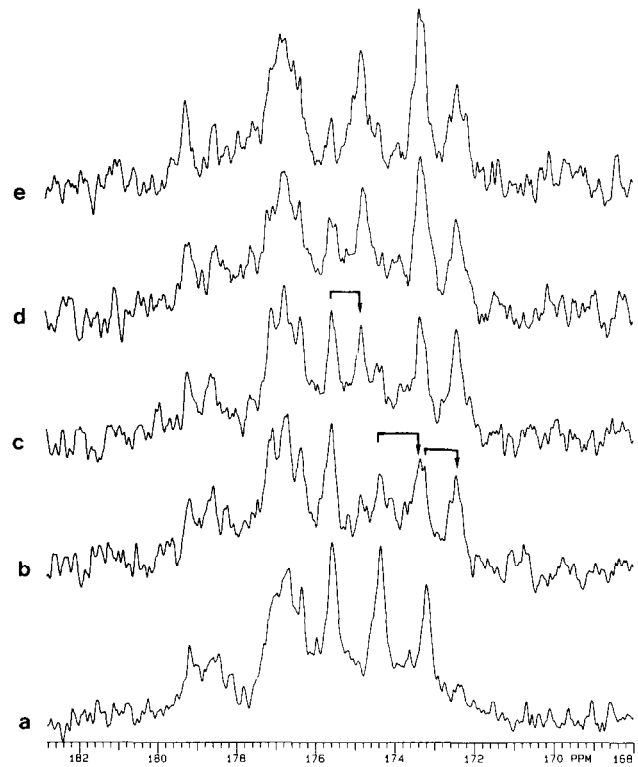

Fig. 4. ^{13}C-NMR spectra recorded at 50°C of (M)BR (8 mg/ml) solubilized in 2.5% n-dodecylmaltoside showing the kinetics of methionine residue oxidation by H_2O_2. The oxidation was monitored directly in the NMR spectrometer in 12 h intervals after addition of H_2O_2 at time 0. (a) before H_2O_2 addition, (b) 0–12 h incubation, (c) 12–24 h incubation, (d) 24–36 h incubation, (e) 36–48 h incubation. The arrows indicate the evolution of the ^{13}C resonances affected by oxidation.

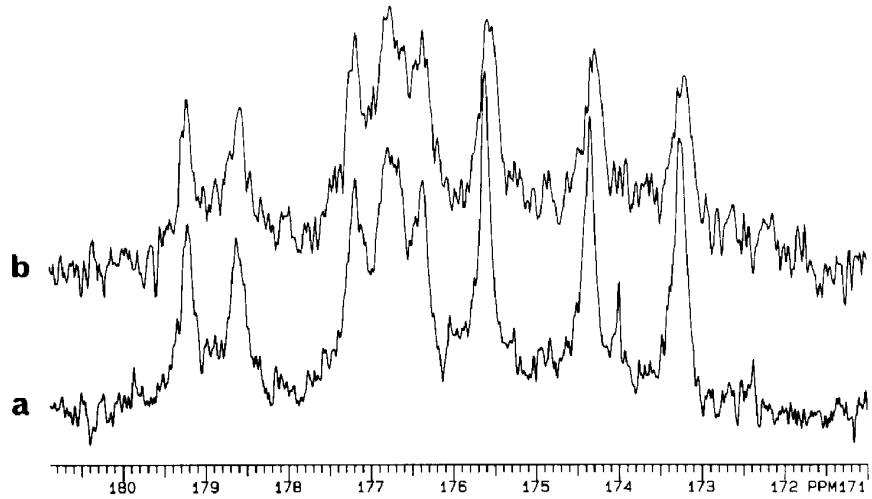

Fig 5. ^{13}C-NMR spectra recorded at 50°C of (M)BR (8 mg/ml) solubilized in 2.5% n-dodecylmaltoside in H_2O–^2H_2O 9:1 (a) and 5:5 (b) Spectra were processed using a 2 Hz line broadening

denaturation. Comparison with the spectrum of the intact protein in Fig. 3a unambiguously attributes the 174.4 resonance to Met^{68} (note that the nearby 174.1 ppm line of the cleaved protein spectrum results from a shift of the 173.3 line due to denaturation and can also be seen with reduced intensity in the spectrum of the non-proteolysed protein).

3.3. *Determination of water exposed methionine residue by H_2O_2 oxidation*

We have used the partially assigned ^{13}C-NMR spectrum of (M)BR for structural investigation. It is well known that hydrogen peroxide can oxidize surface methionine residues in proteins and that such oxidation can be monitored by NMR. The resulting methionine sulfoxide residue has its carbonyl ^{13}C resonance shifted 1 ppm upfield [19]. Fig. 4 shows the evolution of the ^{13}C NMR spectrum of (M)BR in dodecylmaltoside during oxidation of the protein by H_2O_2 performed during the progressive NMR acquisition at 50°C (successive 12 h acquisitions were done). The first noticeable effect is the disappearance of the 173.3 and 174.4 ppm resonances with parallel growing of 2 lines 1 ppm upfield. On the basis of the above sequence-specific assignments, this indicates a water-exposed character of Met^{68} and Met^{163}. A slightly slower effect is the oxidation of a third methionine residues as indicated by the shift of the 175.6 ppm resonance to higher field. The above double-labelling experiments indicated that this line is contributed by either Met^{20} or Met^{32}. Since it would not be possible to construct a realistic folding model of BR with a water-exposed Met^{20}, this third oxidation-sensitive resonance can thus be attributed to Met^{32} (and accordingly the 179.3 ppm line to Met^{20}). The lower oxidation rate of Met^{32} indicates that this residue is slightly buried. All other 6 methionine residues appear to be embedded inside the detergent–protein complex (although other modification of the spectrum were observed at longer times, these were accompanied by progressive denaturation).

3.4. *Measurement of BR amide hydrogen exchange by ^{13}C-NMR*

In order to assess the secondary structure of BR in the vicinity of methionine residues, we have studied the effect of hydrogen–deuterium amide exchange on ^{13}C-NMR spectra of (M)BR in dodecylmaltoside. For this purpose, we have used the DEALS method developed by one of us [8]. This makes us of the fact that the resonance position of a particular ^{13}C peptide carbonyl amino acid residue in a protein is shifted 4–7 Hz upfield upon deuteration of the corresponding amide. Comparison of the ^{13}C-NMR spectrum in H_2O and in H_2O–^2H_2O 1:1 allows one not only to identify exchangeable amide residue but also to estimate the exchange rates. Fig. 5 compares the ^{13}C-NMR spectra of (M)BR recorded under those two conditions, respectively. Both spectra were purposely processed with a low level of line broadening in order to emphasize the linewidth differences. The obvious difference between the two spectra is a broadening and a 0.05 ppm upfield shift of the 3 higher field resonances in the H_2O–^2H_2O 1:1 spectrum. This is due to 50% deuteration of the vicinal amide of the corresponding methionine residues, namely Met^{32}, Met^{68} and Met^{163}. Each broadened line has an about doubled linewidth and thus corresponds to a non-resolved doublet pattern associated with the protonated and deuterated species. This indicates that these 3 amide residues are exchangeable and that the exchange is slow or moderately fast (i.e. lifetime > 400 ms, in the case of a faster exchange would narrower lines be expected [8]). These amides are therefore non-hydrogen bonded or weakly hydrogen bonded. On the other hand, it also appears from Fig. 5 that all other 6 methionine carbonyl

resonances have essentially similar lineshapes in the presence or absence of ^2H_2O. The corresponding amide residues therefore do not exchange their proton at least during the 24 h NMR acquisition time at 50°C. This suggest strong hydrogen bonding of these amide residues. Thus only those methionine residues that were found to be water-inaccessible in the oxidation experiment appear to be involved in stable secondary structure elements.

4. DISCUSSION

The present study confirms that it is possible to obtain high-resolution NMR spectra of bacteriorhodopsin solubilized in the detergent dodecylmaltoside. In the case of amino acid species which are contained in moderate number in BR (i.e. less than 10 residues per BR), selective carbonyl ^{13}C labeling allows one to obtain almost complete resolution of single-residue resonances. Even in the case of amino-acid species which exist in higher number per BR, a fair resolution appears to be available. In the later case, use of BR regenerated from selectively labeled proteolytic fragments [20] might be used to get complete resolution. A consequence of the achieved resolution is the possibility to observe useful NMR parameters that require narrow linewidths such as ^{15}N-induced splittings or ^2H-induced shifts on ^{13}C spectra. This is for example emphasized by the assignment of several resonances of the ^{13}C-NMR spectrum of (M)BR by double-labeling. The present limitations of this approach are not related to spectral resolution. Metabolic scrambling of ^{15}N might be avoided in the future by the selection of aminotransferase-deficient mutants and ambiguities of assignment by protcolysis (as illustrated here) or again by use of cleaved, regenerated BR.

The sequence-specific assignments obtained here could be used in conjunction with H_2O_2 oxidation and ^1H–^2H exchange experiments monitored by NMR in order to obtain structural information on BR. It appears that, among the 9 methionine residues present in BR, 6 are completely buried from the aqueous surface of the protein. These are Met^{20}, Met^{56}, Met^{60}, Met^{118}, Met^{145} and Met^{209}. Consistently, these are characterized by a very slow (i.e. undetectable) exchange rate of their vicinal amide proton which seem to be involved in stable hydrogen bonding. These six methionine residues are located deeply inside the hydrophobic membrane spanning α-helical regions of the protein. On the other hand, our data show that the three remaining methionine side chains are water-exposed. Met^{68} and Met^{163} being slightly more accessible than Met^{32}. All three residues also yield relatively fast amide proton exchange, indicating weak or non-existent hydrogen bonding. This indicates a location of these 3 methionine residues in the hydrophilic loops or at the extremities of the α-helices of BR. All these data confirm the model of BR folding and secondary structure proposed by Henderson et al. [2] on the basis of electron microscopy data. Indeed, according to this model (Fig. 6), Met^{68} and Met^{163} are located in the two largest interhelical loops of BR, Met^{32} is positioned at the cytoplasmic extremity of helix A, while all other 6 methionine residues are found within the interior of α-helices. Note that our data confirm the slightly less accessible character of Met^{32}, implied by this model.

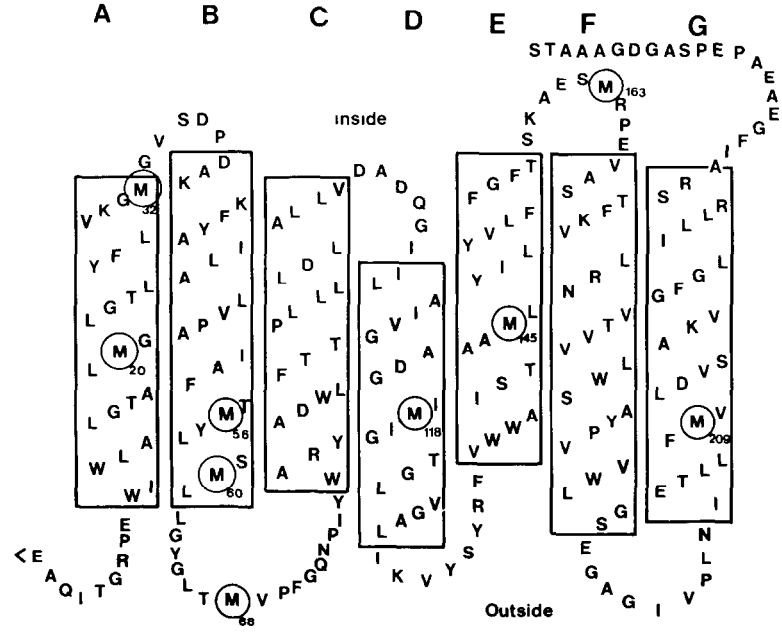

Fig. 6 Secondary structure and transmembrane folding model of BR according to Henderson et al. (2).

Thus high-resolution NMR of detergent-solubilized BR allows one to obtain structural data on the protein and to assess current structure models. The extension of this approach to isotope-assisted multidimensional NMR methods [4] may contribute in the future to the determination of a higher resolution structure of this light-driven proton pump.

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