



Kinetics of an Individual Transmembrane Helix during Bacteriorhodopsin Folding

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The kinetics of an individual helix of bacteriorhodopsin have been monitored during folding of the protein into lipid bilayer vesicles. A fluorescence probe was introduced at individual sites throughout helix D of bacteriorhodopsin and the changes in the fluorescence of the label were time-resolved. Partially denatured, labelled bacteriorhodopsin in SDS was folded directly into phosphatidylcholine lipid vesicles. Stopped-flow mixing of the reactants allowed the folding kinetics to be monitored with millisecond time resolution by time-resolving changes in the label fluorescence, intrinsic protein fluorescence as well as in the absorption of the retinal chromophore. Monitoring specific positions on helix D showed that two kinetic phases were altered compared to those determined by monitoring the average protein behaviour. These two phases, of 6.7 s^{-1} and 0.33 s^{-1} , were previously assigned to formation of a key apoprotein intermediate during bacteriorhodopsin folding. The faster 6.7 s^{-1} phase was missing when time-resolving fluorescence changes of labels attached to the middle of helix D. The amplitude of the 0.33 s^{-1} phase increased along the helix, as single labels were attached in turn from the cytoplasmic to the extracellular side. An interpretation of these results is that the 6.7 s^{-1} phase involves partitioning of helix D within the lipid headgroups of the bilayer vesicle, while the 0.33 s^{-1} phase could reflect transmembrane insertion of this helix. In addition, a single site on helix G was monitored during folding. The results indicate that, unlike helix D, the insertion of helix G cannot be differentiated from the average protein behaviour. The data show that, while folding of bacteriorhodopsin from SDS into lipids is a co-operative process, it is nevertheless possible to obtain information on specific regions of a membrane protein during folding *in vitro*.

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Introduction

Information on the folding mechanisms of α -helical membrane proteins has been difficult to obtain. The last ten years have seen important breakthroughs in the determination of *in vitro* mechanisms through the development of methods to study the kinetics of membrane protein folding.¹ The kinetic approaches for following the folding of transmembrane helical proteins have, however, been limited to monitoring the overall behaviour of the protein through changes in intrinsic protein fluorescence or protein circular dichroism. Here, we use a site-directed labelling approach to focus on a specific transmembrane helix during folding. This

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Abbreviations used: bR, bacteriorhodopsin; bO, bacterio-opsin; CMC, critical micelle concentration; DMPC, L- α -1,2-dimyristoylphosphatidylcholine; DOPC, L- α -1,2-dioleoylphosphatidylcholine; DPOPC, L- α -1,2-dipalmitoleoylphosphatidylcholine; PC, phosphatidylcholine.

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study demonstrates another application for site-specific labelling, which is one of the most powerful methods for probing conformational changes, and particularly transmembrane helix movements, during membrane protein function.²⁻⁷

Bacteriorhodopsin (bR) was the first integral membrane protein to be unfolded and refolded *in vitro*, and has led the way in studies of transmembrane helical protein folding.⁸⁻¹⁰ Most of the methods to probe helical membrane protein folding mechanisms were developed originally on bR, including kinetic, thermodynamic and mechanical approaches to monitor folding or unfolding.¹¹⁻¹⁵ Equally, the methods used to refold bR *in vitro* are those that have since proved successful for other proteins. The central method for refolding bR involves a partially denatured state in sodium dodecyl sulphate (SDS), at a concentration of SDS just above the critical micelle concentration (CMC). The SDS-denatured protein is then diluted into renaturing detergent micelles or lipid vesicles, giving a final concentration of SDS just below the CMC.^{8,9,11,16,17} This method, with only slight modifications, has proved applicable to the potassium KscA channel,¹⁸ the disulphide-binding protein DsbB,¹⁹ the major light-harvesting complex of higher plants LHCII,^{20,21} and DGK, an *Escherichia coli* kinase protein.^{22,23} As a result, kinetic or thermodynamic studies of the folding of these helical membrane proteins has commenced, but bR remains the most intensively studied protein.

bR is a seven transmembrane α -helical protein with a retinal cofactor bound within this helical bundle.²⁴⁻²⁶ The kinetics of folding bR from SDS into detergents and lipids have been studied extensively and folding occurs through a series of identifiable intermediates.^{27,28} Figure 1 shows a simplified reaction scheme that applies to folding into lipid vesicles. The starting state for the reaction, bacterio-opsin (bO) in SDS, has just over half the native helix content.^{9,29} A key apoprotein

intermediate is referred to as I_2 and is present during folding into micelles as well as vesicles.^{9,11,27} There appear to be two I_2 states (I_{2a} and I_{2b}) in lipid vesicles, which can be differentiated in terms of their kinetics and response to bilayer curvature stress and lateral pressure.^{27,30} This suggests there may be different conformations of the protein present in these intermediate states. Moreover the kinetics of formation of the I_2 states appear to be multi-exponential, again indicative of different protein conformations or lipid environments. There may be different protein conformations present in the I_R state, which has the retinal cofactor non-covalently bound to the protein. The structure of the protein in the I_2 state is unknown, although it appears to have native helical content (i.e. equivalent to seven transmembrane helices), together with some native tertiary structure.^{29,31}

The experimental methods that have been used to determine the folding kinetics of bR have involved measuring overall properties of the protein; for example, by following changes in intrinsic protein (mainly from Trp and Tyr) fluorescence or far-UV protein circular dichroism spectra (i.e. secondary structure content). These methods therefore cannot distinguish between the behaviour of different parts of the protein. Nor is it possible to ascertain whether multi-exponential kinetics arise from Trp residues in different regions of the protein reporting on different behaviour in those regions, or from different protein molecules that are in different conformations. In addition, assigning changes in overall protein fluorescence to particular events is difficult. An increase in Trp fluorescence can result from either a more hydrophobic lipid/detergent environment, or folding the Trp into a more hydrophobic protein interior, or from a reduction in quenching of the Trp. Attaching an individual fluorescence label at a specific site in the protein overcomes some of these difficulties.

bR was chosen for this study partly because of the extensive mechanistic detail available on bR folding, but also because there are fairly stringent requirements for site-specific labelling, which this protein fulfils. Labels are best introduced by attachment to Cys SH groups, and thus single Cys mutants of the protein are needed.^{32,33} Furthermore, the labelled Cys mutants should exhibit wild-type folding and functional properties. bR was the first membrane protein for which the site-specific Cys labelling strategy was demonstrated.³⁴ The protein has no native Cys residues and it has proved possible to introduce single Cys residues at many sites throughout the protein and to label them without significant effects on the overall folding and function of the protein.³⁵⁻³⁹ Most of the previous work on bR has involved the attachment of spin labels rather than fluorescence labels, and has been aimed at elucidating conformational changes of the protein during its functional photocycle.² In the case of bR, the protein can be labelled in the SDS state and folded to a functional state in detergent micelles. Whilst the folding kinetics have not been

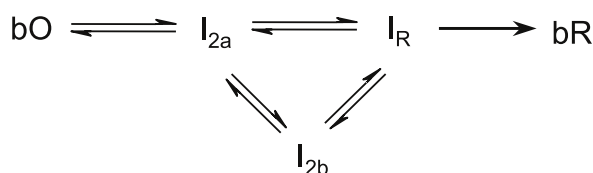


Figure 1. A reaction scheme for folding SDS-bO to bR in lipid vesicles.²⁷ bO is the SDS-denatured bO starting state that has just over half the native helix content, while bR is the correctly folded state with retinal covalently bound. The central aspects of the scheme are the two I_2 states, I_{2a} and I_{2b} , which are thought to have native secondary structure but different tertiary structure, possibly due to lipid lateral pressure effects. Retinal binds to both I_2 states to give an intermediate I_R with retinal non-covalently bound. There may be additional states; I_1 (between bO and I_2 , for example to model the observed rate k_{obs}), more than one I_R state⁶³ and I_3 (between I_R and bR). However, these have been omitted for clarity. (It is possible to invoke other schemes, with additional branched or parallel reactions.)

studied, this earlier work on bR shows the sites on the protein where labels can be attached successfully without affecting the final folded state. The majority of studies on this (and other membrane proteins for which the method has been used) have also involved working in detergent micelles rather than lipid bilayers. Nevertheless, it is possible to work in bilayer systems and to introduce labels that will be embedded within the bilayer.⁴⁰ We have chosen to focus on folding bR into lipid bilayer vesicles, since the protein is more stable in vesicles; as the bilayer provides a more robust solvent environment. In addition, we have developed bilayer vesicles in which the folding kinetics can be controlled. Micelles, in contrast to bilayers, are far more likely to alter their structure and solvate the fluorescence label during folding, which in turn will affect the protein-solvent interactions and folding dynamics throughout the folding process. However, working in lipid vesicles presents different problems. Labelling a site on the protein that will be transmembrane in the folded state can be difficult in a vesicle; a label has to be chosen that will partition into the vesicle bilayer, whilst not affecting the insertion kinetics of the protein or particular transmembrane segment. We show that it is possible to choose an appropriate fluorescence label that inserts into lipid bilayers and does not have significant effects on the folding kinetics. We have focussed on labelling protein sites that face the lipids (so the label will not interfere with the protein interior) and have predominantly chosen sites that are already known to function when labels are attached, albeit spin labels in detergent micelles. Fluorescence labels tend to be larger than spin labels and thus there is greater potential for them to interfere with folding kinetics. We have chosen a relatively small fluorescence label, monochlorobimane (see Figure 2), which has properties similar to monobromobimane that has been used successfully in other membrane protein studies.⁴¹ Monochlorobimane partitions into lipid bilayers and shows a greater selectivity towards reactions with thiol groups than monobromobimane.

Transmembrane helix D of bacteriorhodopsin was targeted in this study. In terms of sequence position, helix D is a central helix, and single Cys residues have been introduced and labelled (with spin labels) throughout this helix,⁴² thus showing

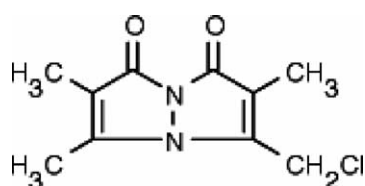


Figure 2. The structure of monochlorobimane. The thiol (SH) group of Cys reacts with monochlorobimane resulting in the formation of a covalent bond between the carbon atom, to which chlorine is attached, and the Cys sulphur atom, accompanied by the loss of chloride.

those sites that can be successfully labelled (in detergent micelles). Moreover, labelling along the helix affords the opportunity to probe the behaviour of the whole helix during folding. We also report on Cys mutants at sites in helices A and G. These are the first and last helices of bR and there is evidence to suggest they may behave differently during folding, with helix G forming and inserting later during folding. In the absence of existing, appropriate Cys mutants on these helices A and G, potential sites were chosen from the crystal structure; i.e. those that face away from the protein interior. For each Cys mutant, we have assayed its ability to fold, both unlabelled and labelled, into micelles as well as lipid vesicles. Mixed L- α -1,2-dimyristoylphosphatidylcholine (DMPC)/Chaps micelles are used as an initial check on the ability of the labelled proteins to fold, as this has been the most extensively used micelle system for folding bR and works well for most bR mutant proteins.^{30,35,37,43–45} L- α -1,2-Dioleoylphosphatidylcholine (DOPC) vesicles are used, since this is a readily available synthetic lipid, a single-component lipid vesicle simplifies data interpretation and we have previously studied bR folding in this system.¹⁷ The folding kinetics of those labelled proteins that fold successfully into vesicles has then been studied. The focus of these kinetic studies is the formation of the key intermediates, I_{2a} and I_{2b}. Mixing the SDS state with DOPC vesicles, in the absence of retinal, can readily follow the folding from the SDS-denatured state to give these intermediates. As described in detail in earlier reports, this enables the kinetics of I_{2a} and I_{2b} formation to be monitored without the complications of the later retinal binding reactions.^{27,46}

Results

Helix D

Folding yields of labelled and unlabelled proteins

Five positions, all of which have previously been spin-labelled,⁴² were chosen on helix D for the attachment of bimane (see Figure 3). A single Cys was introduced in turn at each of these positions to give the following bR mutant proteins: G113C, G116C, G120C, V124C and G125C. Each mutant was labelled with bimane and the folding yield of each mutant protein (labelled and unlabelled) was determined in both DMPC/Chaps micelles and DOPC vesicles (Figure 4). Proteins were folded from SDS into the micelles or vesicles and the yield determined from the amount of purple bR chromophore that formed, as described.^{17,27,46} Figure 4 shows there is a slight reduction in the yield of the mutant proteins in micelles or vesicles, when compared to wild-type yields (of approximately 95% in micelles and 70% in vesicles). The bimane label affected the yield significantly in only two cases. The yields of labelled V124C-bimane and

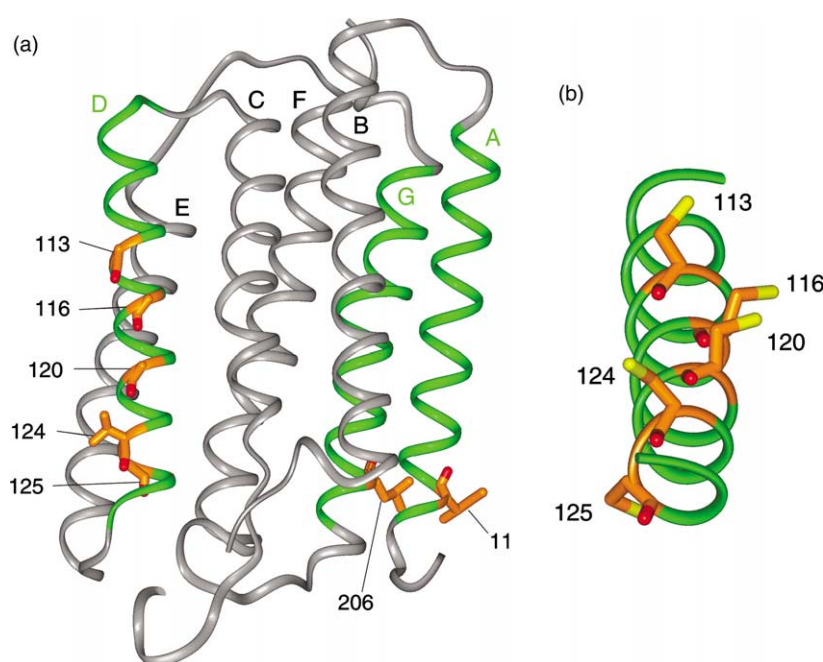


Figure 3. Ribbon diagrams of bacteriorhodopsin showing the labelling sites. (a) Structure of bacteriorhodopsin constructed using INSIGHTII and the co-ordinates reported by Luecke *et al.* (1c3w).²⁵ The labelling sites on helices A, D and G are shown in orange, with backbone O, C and side-chain atoms present, the three helices are shown in green. The extracellular side of the protein is at the bottom. (b) A representation of helix D, showing that positions 116 and 120 are on a different face to the other labelling positions on the fully folded helix. Constructed using co-ordinates as in (a), but with residues 113, 116, 120, 124 and 125 changed to cysteine and energy minimisation in INSIGHT performed on the side-chain rotamers to illustrate that in an isolated helix the cysteine side-chain positions shown in the Figure are plausible.

G125C-bimane were lower (about half) than the corresponding unlabelled protein in vesicles. The yields of unlabelled V124C and G125C, however, were higher in vesicles than micelles, and thus the folding yields of V124C-bimane and G125C-bimane were similar in both micelles and vesicles.

Folding kinetics

In order to determine whether the mutants fold like wild-type protein, the folding kinetics of each labelled and unlabelled protein were determined by following changes in intrinsic protein fluorescence. Each protein was folded from SDS into DOPC vesicles to give the I_2 intermediate, as described.^{27,29,46} The folding was initiated by stopped-flow mixing and the changes in protein fluorescence during folding were followed over time. Table 1 shows that the observed rates and amplitudes for the Cys mutants were similar to wild-type in each case, showing that each mutant folds according to the same reaction scheme as wild-type (i.e. Figure 1). The fastest two observed rates, $k_{\text{obs}1}$ and $k_{\text{obs}2}$, relate to mixing of SDS and DOPC (whether one or two phases are observed for this process depends on the data density and signal-to-noise ratio for that particular measurement).^{11,27,46,47} The phases $k_{\text{obs}3}$, $k_{\text{obs}4}$ and $k_{\text{obs}5}$ all relate to the formation of I_{2a} (possibly *via* an intermediate I_1), while $k_{\text{obs}5}$ reflects the slower formation of I_{2b} . The $k_{\text{obs}5}$ phase was observed for all mutants; however, actual values are not given in Table 1 for V124C and G125C. The data quality over longer times from stopped-flow measurements was lower for these two mutants than the others, which made precise resolution of rate constants difficult.

The lower signal-to-noise ratio could indicate either that there is less of the $k_{\text{obs}5}$ phase, the rate constant is slower, or that the I_2 state is slightly less stable in these Cys mutants. It is hard to assign this to a significant difference in $k_{\text{obs}5}$ for V124C and G125C, since this slow rate is sensitive to changes in vesicle preparations over time and is difficult to measure precisely. In addition, this observed rate depends on the equilibrium between bO and all the I_{2a} and I_{2b} states, which in turn depends on the stability of I_{2b} , and both this equilibrium and stability could be altered slightly for different mutants.^{30,48,49}

No significant change was seen in the protein fluorescence kinetic parameters for the bimane-labelled mutants (data not shown), with respect to their unlabelled counterparts (data in Table 1). The fact that V124C-bimane and G125C-bimane show wild-type folding rates, despite having lower folding yields in vesicles than unlabelled V124C and G125C (see Figure 4(b)) shows that the proportion of these labelled mutants that do fold to a functional state, do so according to a mechanism similar to that of the wild-type protein. The reduced yield is therefore most likely to arise from some of the labelled protein not incorporating into DOPC vesicles, but remaining in SDS and thus not contributing to the folding kinetics. We noted earlier that reduced folding yields arise from protein remaining in SDS.⁴⁸ The signal-to-noise ratio of the protein fluorescence kinetic data for some of the labelled mutants was lower than for the unlabelled mutant proteins. This makes it harder to resolve the different kinetic parameters accurately, particularly since there are several kinetic phases present. The lower signal-to-noise ratio of the labelled mutant data could arise from the relatively

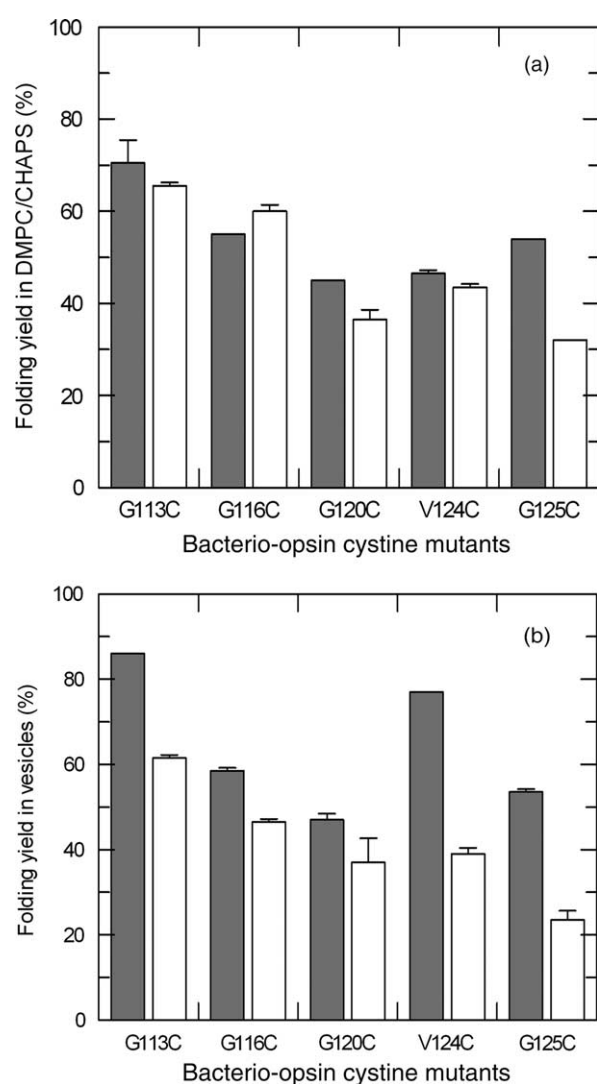


Figure 4. Folding yield of labelled and unlabelled cysteine mutants in DMPC/Chaps micelles and DOPC vesicles. Yields of helix D cysteine mutants folded into (a) DMPC/Chaps micelles and (b) DOPC vesicles. Filled bars represent folding yields for unlabelled proteins, while open bars are for bimane-labelled proteins. Error bars are for the first standard deviation between measurements on two different samples. We have reported extensively on errors before, reproducibility between different protein preparations is the most relevant here, with errors in wild-type protein yields of $88(\pm 16)\%$ in DMPC/Chaps⁴⁶ and $70(\pm 4)\%$ in PC lipid vesicles.²⁷

low folding yields (for example for G120C-bimane, V124C-bimane and G125C-bimane), since the main contribution to the observed folding kinetics comes from the protein that can fold successfully. The protein fluorescence intensity may also be altered by interaction with bimane.

Figure 5(a) shows the change in bimane fluorescence on folding G113C-bimane from SDS into DOPC vesicles. There is a small blue shift in the emission band, from 464 nm to 463 nm accompanied by an increase in fluorescence intensity. These changes are indicative of the label

moving into a more hydrophobic environment,⁵⁰ probably as a result of bimane entering the lipid bilayer of the vesicles. Indeed, a blue shift in bimane fluorescence has been found to correlate well with the solvent-accessibility of bimane.⁵⁰ However, the spectra in Figure 5(a) may be complicated by Trp quenching of bimane, which seems to occur at short distances through photoinduced electron transfer.⁵¹

The change in bimane fluorescence intensity upon folding was monitored by time-resolving bimane emission above 420 nm. Table 2 shows the kinetics parameters obtained from bimane fluorescence data, compared to those (shown in the top row) obtained from the intrinsic fluorescence measurements on wild-type.²⁷ Time-resolved data were collected over several timescales. A rapid decrease is observed in the bimane fluorescence, with a rate that corresponds to the initial mixing of the SDS micelles and DOPC vesicles. This is followed by a multi-phasic increase in bimane fluorescence with observed exponential rates ($k_{\text{obs}2}$, $k_{\text{obs}3}$, $k_{\text{obs}4}$ and $k_{\text{obs}5}$) similar to those observed in intrinsic protein fluorescence. Example data over 50 s are shown in Figure 5(b) for G116C-bimane. Table 2 shows that bimane fluorescence data from each mutant exhibit kinetic parameters similar to those observed in intrinsic protein fluorescence during wild-type folding, except in the case of G116C-bimane and G120C-bimane, where $k_{\text{obs}2}$ is missing. A difference across the set of mutants is observed also in the bimane fluorescence for the amplitude, A_3 , of the phase $k_{\text{obs}3}$, which increases on going from G113C-bimane to G125C-bimane (see Figure 6). A_3 is particularly large for G125C-bimane. These changes are reflected in the percentage contribution of the amplitudes of A_3 to the observed bimane fluorescence increase (i.e. the sum of bimane fluorescence amplitudes A_2 , A_3 , A_4 and A_5). Thus, A_3 for G113C-bimane contributes only 6% of the increase, compared to a 55% contribution in the case of G125C-bimane. The relative contribution of A_3 for the former, G113C-bimane, of 6% is similar to that of wild-type, ebO, intrinsic protein fluorescence, where A_3 contributes 9% of the corresponding protein fluorescence increase (i.e. the sum of protein fluorescence amplitudes A_2 , A_3 , A_4 and A_5).

No other significant change or trend was observed in bimane fluorescence for either the rate or amplitude of $k_{\text{obs}4}$ (which represents the slower stage of formation of I_{2a}) or $k_{\text{obs}5}$ (which represents formation of I_{2b}).

In summary, two differences can be seen in the bimane kinetic parameters as compared to those of intrinsic protein fluorescence: (i) the $k_{\text{obs}2}$ phase is missing from the bimane fluorescence at sites 116 and 120; (ii) there is an increase in the $k_{\text{obs}3}$ phase along helix D, with a large A_3 for bimane fluorescence at site 125.

Helices A and G

Sites were selected to attach a label to helix A and helix G. These were towards the extracellular side of

Table 1. Experimentally observed rates and amplitudes determined from protein fluorescence data during folding of bO cysteine mutants in DOPC vesicles, in the absence of retinal (bO $\rightarrow$ I₂)

Proposed origin of phase		Experimentally observed rate constants, k_{obs} (s ⁻¹), with amplitudes shown in parentheses					
		$k_{\text{obs}}1^a$	$k_{\text{obs}}1^a$	$k_{\text{obs}}2$	$k_{\text{obs}}3$	$k_{\text{obs}}4$	$k_{\text{obs}}5$
		Vesicle mixing		I _{2a} formation			I _{2b} formation
	ebO ^b	* ^a	67	6.7	0.33	0.032	0.0019
Helix D	G113C	290 (−0.008) ^c	25 (−0.004)	2.4 (−0.002)	0.19 (−0.002)	0.029 (−0.008)	0.0016 (−0.02)
	G116C	350 (−0.006)	43 (−0.005)	2.4 (−0.002)	0.16 (−0.003)	0.021 (−0.008)	0.0054 (−0.010)
	G120C	160 (−0.005)	* ^a	11 (−0.007)	0.26 (−0.002)	0.024 (−0.002)	0.0024 (−0.002)
	V124C	310 (−0.002)	* ^a	5.4 (−0.003)	0.58 (−0.003)	0.054 (−0.002)	Phase present ^d
	G125C	190 (−0.004)	* ^a	11 (−0.003)	0.27 (−0.002)	0.038 (−0.0008)	Phase present ^d
Helix G	L206C	223 (−0.007)	21 (−0.007)	7.4 (−0.01)	0.33 (−0.002)	0.040 (0.02)	0.0046 (−0.02)

^a Both rates $k_{\text{obs}}1$ and $k_{\text{obs}}1$ are assigned to the rate of stopped-flow mixing of SDS and vesicles. These rates are hard to assign precisely due to variations in lipid preparations over time. Whether one or two rates are observed depends on the data density and signal-to-noise for that particular measurement, and the correlation of the exponential fitting functions means the two rate constants cannot be satisfactorily separated. Thus, in some cases, data are represented adequately by one exponential, as shown by a single value for $k_{\text{obs}}1$ or $k_{\text{obs}}1$ and *.

^b Values for wild-type protein are shown for comparison. These are taken from Allen *et al.*²⁷ as these are the most precisely determined values for wild-type protein. Details and errors are given by Allen *et al.*²⁷ These values are from measurements in DOPC vesicles, whereas all mutant data are from measurements in DPOPC vesicles. No significant difference was found in the kinetics parameters during folding of ebO into DOPC or DPOPC vesicles.

^c Amplitudes are normalised to 1 μ M bO protein and are negative, as they represent an increase in fluorescence.

^d Rate constants were estimated from data collected over 2000 s, using stopped-flow mixing to initiate folding. Rates over this time are difficult to measure, both due to back mixing of the stop volume into the cuvette as well as changes in vesicles over time. Experience with vesicle measurements over this time (as well as checks with time-resolved absorption and fluorescence measurements initiated by mixing in a cuvette without stop flow) indicate the $k_{\text{obs}}5$ phase is present in all mutants. However, in the case of V124C and G125C the slightly worse signal-to-noise ratio and reproducibility over the long time-scale meant that it was not possible to resolve a meaningful rate for the phase.

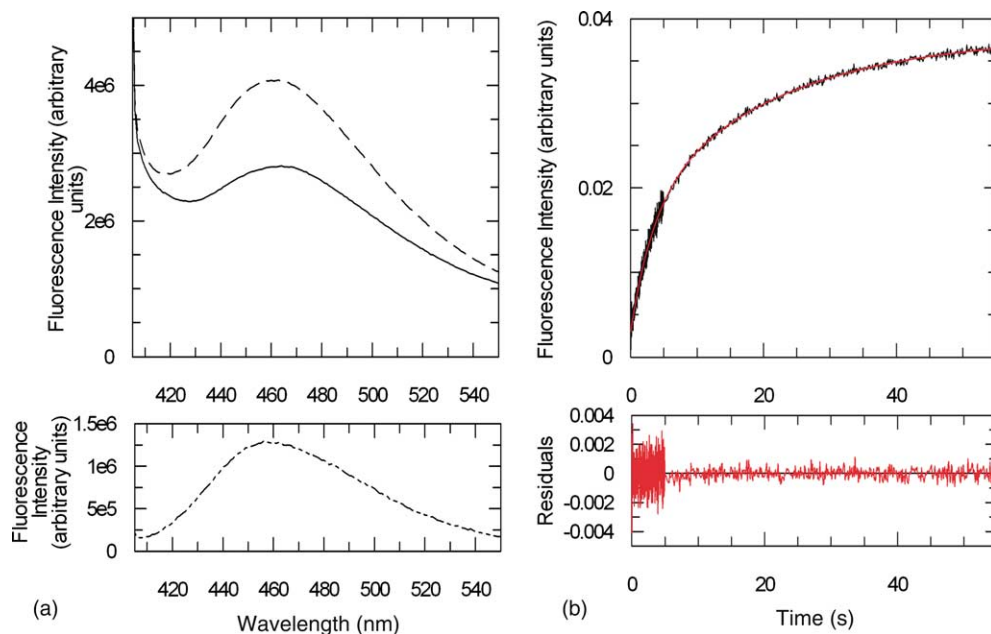


Figure 5. Changes in bimane fluorescence during folding to bacteriorhodopsin. (a) Bimane fluorescence spectra of 4 μ M G113C-bimane of the SDS-denatured starting state (continuous line) and the folded bR state, 30 min after refolding into 2% DOPC vesicles (broken line). Bimane was excited at 384 nm with an excitation bandwidth of 1 nm and emission bandwidth of 10 nm. The lower panel shows the difference between the two fluorescence spectra (i.e. refolded DOPC-SDS-denatured). (b) Changes in bimane fluorescence during folding from bO to bR in DOPC vesicles for G116C-bimane. The red curve represents a two-exponential fit to the data, for which the residuals are shown below and that which resolved the following parameters: $A_3 = -0.02$, $k_{\text{obs}3} = 0.29 \text{ s}^{-1}$, $A_4 = 0.02$ and $k_{\text{obs}4} = 0.048 \text{ s}^{-1}$. A total of 4000 data points were collected, split equally over time-scales of 5 s and 50 s. The final protein concentration was 4 μ M, but the data shown are normalised to 1 μ M final protein, and are the average of three separate data sets. Data were collected over other time-scales to resolve the other kinetic parameters.

Table 2. Experimentally observed rates and amplitudes determined from bimane fluorescence data during folding of bimane-labelled, bO cysteine mutants in DOPC vesicles, in the absence of retinal (bO→I₂)

		Experimentally observed rate constants, k_{obs} (s ⁻¹), with amplitudes shown in parentheses					
		$k_{\text{obs}}\hat{1}$	$k_{\text{obs}}1$	$k_{\text{obs}}2$	$k_{\text{obs}}3$	$k_{\text{obs}}4$	$k_{\text{obs}}5$
Proposed origin of phase		Vesicle mixing		I _{2a} formation		I _{2b} formation	
	ebO ^a	* ^b	67	6.7	0.33	0.032	0.0019
Helix D	G113C-bimane	470 (0.09) ^a	32 (0.001)	1.6 (−0.004)	0.27 (−0.004)	0.056 (−0.002)	0.0078 (−0.01)
	G116C-bimane	49 (0.07)	18 (0.01)	Phase absent	0.11 (−0.002)	0.048 (−0.015)	0.0048 (−0.02)
	G120C-bimane	530 (0.1)	33 (0.003)	Phase absent	0.43 (−0.003)	0.060 (−0.007)	0.0004 (−0.006)
	V124C-bimane	390 (0.05)	29 (0.03)	1.2 (−0.008)	0.364 (−0.01)	0.05 (−0.006)	0.0024 (−0.02) ^c
	G125C-bimane	36 (0.05)	25 (0.02)	1.4 (−0.007)	0.19 (−0.03)	0.038 (−0.01)	0.00067 (−0.0004) ^c
Helix G	L206-bimane	94 (0.06)	* ^d	2.6 (−0.006) ^e	0.29 (−0.004) ^e	0.036 (−0.03) ^e	0.0046 (−0.01) ^e

^a Values taken from Allen *et al.*²⁷ for wild-type are shown for comparison, see footnote^b to Table 1.

^b Amplitudes are normalised to 1 μM bO protein with a further correction for the percentage of bimane label bound to the protein.

^c From measurements using a steady-state fluorimeter, following mixing into a cuvette without stopped-flow. All other $k_{\text{obs}}5$ values quoted are from stopped-flow data.

^d Data were represented adequately by one exponential, as shown by a single value for $k_{\text{obs}}\hat{1}$ or $k_{\text{obs}}1$ and *, see footnote^a to Table 1.

^e Data were collected with different excitation light intensity and thus to account for this as accurately as possible, the total fluorescence changes was normalised to that of wild-type (and to folding yield), as well as to protein concentration.

helices A and G, giving the mutants I11C on helix A and L206C on helix G (see Figure 3(a)). These two positions face out from the protein interior and thus are likely to be amenable for labelling without affecting the protein structure. The two mutants were successfully expressed, purified and folded to give yields of 72% and 53% for I11C in DMPC/Chaps and lipid vesicles, respectively, and 63% and 69% for L206C in DMPC/Chaps and lipid vesicles, respectively. Both mutants were also labelled successfully with bimane and folded to a yield similar to that of the unlabelled mutant in DMPC/Chaps micelles; 60% for I11C-bimane and 63% for L206C-bimane. However, only L206C-bimane could be folded into DPOPC vesicles successfully (with 74% yield), while no formation of a purple bR

chromophore could be detected on transferring I11C-bimane from SDS into lipid vesicles.

The kinetics of folding into lipid vesicles of the bimane label of L206C-bimane were very similar to those determined from intrinsic protein fluorescence measurements of wild-type, ebO protein. All rates $k_{\text{obs}}2$, $k_{\text{obs}}3$, $k_{\text{obs}}4$ and $k_{\text{obs}}5$ were observed with no significant change in the value of the observed rate, in both the intrinsic protein fluorescence of the L206C mutant as well as the bimane fluorescence of L206-bimane (see Tables 1 and 2, bottom row). The relative contributions of the amplitudes of the bimane kinetic phases were similar to those determined from intrinsic protein fluorescence measurements. Thus, for example, the percentage contributions of A_3 , A_4 and A_5 to the overall increase in fluorescence (i.e. the sum of amplitudes A_2 , A_3 , A_4 and A_5) resolved from the bimane data for L206C-bimane were 15%, 13% and 46%, respectively. These are similar to those for wild-type intrinsic protein fluorescence data for which A_3 , A_4 and A_5 were resolved as 9%, 27% and 46%, respectively. (The differences in A_2 and A_3 in the two data sets are probably not significant and most likely represent correlation of the exponential components during fitting).

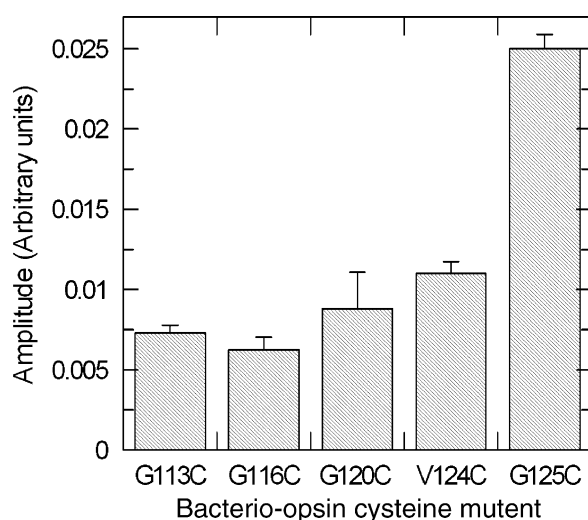


Figure 6. Increase in the amplitude A_3 of the kinetic phase, $k_{\text{obs}}3$ along helix D. Errors shown are the first standard deviation from three different samples.

Discussion

Further insight into the folding mechanism of bR and insertion of helix D

Individual fluorescence labels have been attached successfully throughout helix D and used to report on the kinetic behaviour of this helix during folding of bR from SDS into lipid vesicles. Similarly, one position on helix G has been labelled and folded into vesicles, allowing this site on helix G to be followed during folding. These sites on helix D and

helix G have been followed during apoprotein folding to a key intermediate I_2 . Overall, the folding kinetics of these specific regions of bR are similar to those for the protein as a whole. Kinetic phases are observed for each individual site similar to those resolved when following changes in the intrinsic protein fluorescence, which reports on several sites across the protein. This shows that the folding of bR, from SDS into lipids, is extremely co-operative and it is not possible to identify distinct folding patterns for different regions of the protein during formation of I_2 . This is consistent with earlier reports on the folding behaviour of bR mutants, where only subtle changes in the folding kinetics to I_2 have been observed.^{31,30,49}

There are subtle, but significant differences in the kinetic parameters resolved during overall protein folding (from intrinsic protein fluorescence) as compared to those resolved at specific sites on helix D (from bimane fluorescence). These are centred on two kinetic phases; $k_{\text{obs}2}$ and $k_{\text{obs}3}$. The most significant of these differences in protein and bimane fluorescence is the absence of the $k_{\text{obs}2}$ phase in the bimane fluorescence data at two positions in the middle of helix D; 116 and 120. This $k_{\text{obs}2}$ phase has previously been assigned to an early stage in the formation of I_{2a} and may reflect the formation of an intermediate I_1 . The nature of an I_1 intermediate remains elusive but it seems to represent protein conformational changes partly associated with a change in solvent.^{10,28} It is not immediately apparent why two sites (116 and 120) that reside in the middle of a transmembrane helix would be lacking this phase in the bimane fluorescence data, but not the intrinsic protein fluorescence. The explanation may, however, lie in the orientation of the positions 116 and 120 on helix D. Figure 3(b) shows that although all the labelling sites face in one general direction (i.e. primarily towards the lipids), the side-chains of residues 116 and 120 are on a different face of the helix to those of 113, 124 and 125, and thus could experience different solvent environments. For example, it is conceivable that this helix could associate with the headgroup region of a lipid bilayer, with residues 113, 124 and 125 partitioning into the headgroups, while 116 and 120 face out to the aqueous phase or to SDS. This would suggest that $k_{\text{obs}2}$ involves the partitioning of helix D into the lipid headgroups, together with association/rearrangement of the remainder of the protein with the lipid vesicles, and that helix D is either already formed or forms during this process. This partitioning event of helix D does not involve the initial encounter of the SDS-denatured protein with the DOPC vesicles, since the initial mixing of the protein-SDS micelles and DOPC vesicles occurs with an apparent rate constant tenfold faster than $k_{\text{obs}2}$, of the order of 67 s^{-1} .

Another difference that can be seen from the fluorescence label studies on helix D as compared to the folding behaviour of the overall protein is in the amplitude, A_3 , of the kinetic phase $k_{\text{obs}3}$.

A_3 increases in the bimane fluorescence data as the label is introduced at different sites down the helix, towards the extracellular side. No such distinct trend is observed for A_3 when monitoring the overall protein behaviour of the unlabelled or labelled Cys mutants, nor in any other of the kinetic phases resolved during protein or bimane fluorescence experiments. Thus, this increase in the bimane A_3 down helix D is not due to more proteins folding and undergoing the reaction represented by A_3 , since that would also increase the value of A_3 resolved in protein fluorescence experiments as well as the amplitudes of other kinetic phases. A more likely explanation is that the increase in A_3 reflects an increase in the hydrophobic environment down helix D during this stage of folding. This would occur if the helix moves from SDS (or the lipid headgroups) into the more hydrophobic chain region of the DOPC bilayer. There are two possible causes for such an increase in hydrophobicity: either helix D rotates to expose the outer face of helix D (where the labels are sited) from SDS to DOPC lipids, or helix D "inserts" from SDS into the DOPC lipids. Insertion as a transmembrane helix is an attractive explanation, considering the trend down the helix and the model proposed above, where $k_{\text{obs}2}$ represents helix D partitioning into the lipid headgroups. The most straightforward model would then be that helix D is partly inserted in the headgroups of the outer layer of the vesicles bilayer ($k_{\text{obs}2}$) and then inserts across the bilayer ($k_{\text{obs}3}$), with residue 125 and the extracellular face of the protein residing on the inside of the bilayer, thus giving an "inside-out" orientation of the protein. This is in line with other reports on bacteriorhodopsin reconstitution or insertion into lipids, where it frequently adopts such an inside-out orientation.¹⁶ This orientation, with the extracellular surface inside, means that bR pumps protons into the vesicle. Preliminary FTIR and proton pumping measurements (data not shown) on protein folded from SDS into PC vesicles, agrees with this orientation and shows that the protein pumps protons, and vectorial pumping into the vesicle can be detected.⁵²

We have previously proposed two models for protein conformational changes that could be occurring during formation of I_2 .^{27,31,48,53} One model suggests that I_2 formation involves the packing of pre-formed cores of most of the seven helices of bacteriorhodopsin, followed by helix formation at the helix ends. The second model proposes that I_2 formation involves the packing of five pre-formed helices, A–E, and this is followed by formation and insertion of the last two helices, F and G. The work presented here shows that a label on the extracellular side of helix G exhibits folding kinetics that are similar to those seen for the whole protein, with no significant differences in rates or relative amplitudes. Since Trp residues dominate the intrinsic protein fluorescence, of which there are none on helix G, the kinetics of helix G are very similar to those of the rest of the protein. Hence,

the second model where G inserts and folds later during folding is unlikely (or the process is more co-operative and a final folding/insertion of G cannot be distinguished). If helix G did insert during I_2 formation, a larger relative amplitude for a phase reflecting I_2 formation would result (i.e. in A3, A4 or A5), as observed, for example, for A3 when monitoring position 125 on helix D.

The results presented here suggest that helix D inserts during I_2 formation, and thus this should be incorporated into the first model. Previously, we suggested that the rate-limiting formation of I_2 involved the orientation and packing of the cores of helices A to G, followed by folding of the helix ends (corresponding to ~ 30 amino acid residues folding to helical structure, or "capping" of two or three amino acid residues at the ends of each helix).²⁹ We suggested also that the lateral pressure exerted on the protein by the lipid chains affected the helix packing, and that this could be responsible for

the observation of two forms of I_2 in lipid vesicles, I_{2a} and I_{2b} .^{27,48} We can now refine this model, as in Figure 7, and propose that I_2 formation involves insertion of helix D as well as helix formation at the ends and helix packing. Such a model implies also that helix E forms/inserts with D, perhaps as a helix hairpin. The chain pressure of the lipids could affect this helix insertion.^{48,54} Figure 7 is only one possible model and, in view of the fact that there is no definitive information on helices other than D during I_2 formation, helices A, B, C, E, F and G are drawn arbitrarily in Figure 7, albeit on the basis of the previous models. Furthermore, the molecular details and interactions that SDS makes during folding into lipids are unknown.^{27,48} In the simplest case, the model in Figure 7 relates primarily to protein conformational changes occurring during the formation of I_{2a} . No significant change in the amplitude of the rate associated with I_{2b} formation (k_{obs5}) was found in any of the site-specific labelling

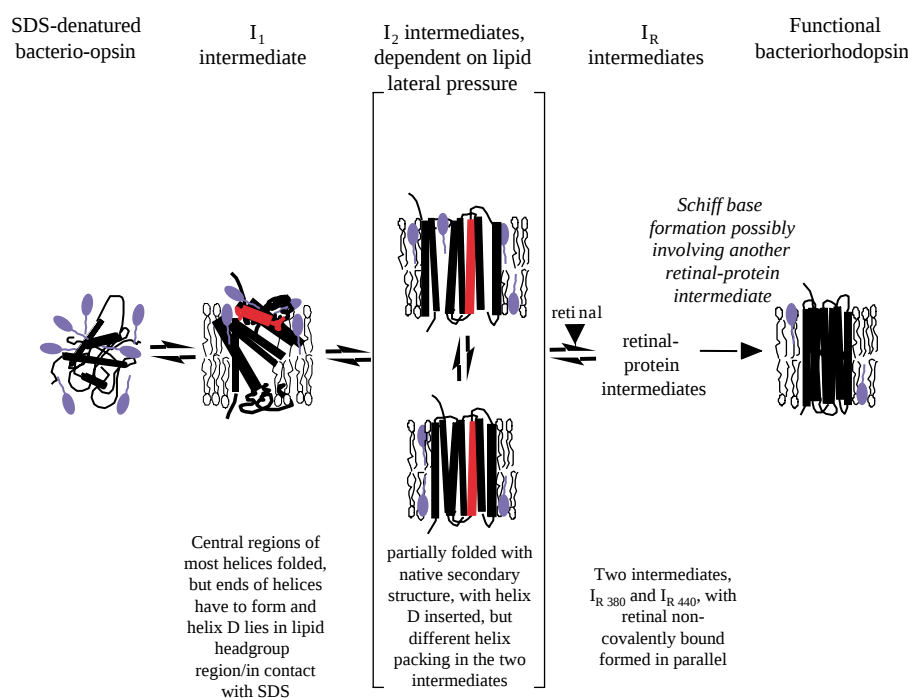


Figure 7. One possible scheme for the folding of bR in lipid bilayer vesicles. The apoprotein, bO, folds from a state in SDS to give the I_2 states (I_{2a} and I_{2b}),²⁷ both of which are shown here with seven transmembrane helical structure. Retinal then binds and this eventually results in formation of bR. Apoprotein folding to I_2 proceeds *via* another intermediate, I_1 (reflected by k_{obs2}), which involves partial formation and insertion of helix D (shown in red) into the lipid headgroup region, with residues 116 and 120 facing out to the SDS or aqueous phase. The later stage of I_2 formation (reflected by k_{obs3}) is suggested to involve helix extension, tilting and packing as well as insertion of helix D.^{27,31,48,53} The difference in the two I_2 states is unknown, but seems to be dependent on the lateral pressure exerted on the helix bundle by the lipid chains.^{47,48} This lateral pressure could affect helix insertion or helix packing. Although we show only a difference in helix packing between I_{2a} and I_{2b} , there could also be a difference in the number of transmembrane helices. SDS molecules are shown in blue. There are no data on the position of SDS during folding; however, it is very likely that all the SDS is incorporated into the bilayer at the end of folding.⁴⁸ The structure proposed here for I_1 is only a suggestion. We suspect that both helices A and B are largely formed at this point, but the relative orientation of the helices is unknown and there may be other helices located in the headgroup region as well as helix D. Moreover, it is not known how much SDS is bound to the protein at this point or inserted into the bilayer, or how this affects the nascent helix bundle. This reaction scheme is based on our earlier suggestions^{1,10,17,29,48,53} but is nevertheless only one possible scheme that, for clarity, does not include all kinetic stages (such as additional retinal, protein intermediates).⁶³

studies down helix D; thus, the insertion of helix D occurs during formation of I_{2a} and not during formation of I_{2b} . However, it is difficult to make a definitive assignment of particular observed rates to actual, intrinsic reaction scheme rates and one scheme (Figure 1) that fits the data includes both forward and reverse rates between I_{2a} and I_{2b} , which are on parallel rather than sequential paths.²⁷ The diagram in Figure 7 does not differentiate between parallel and sequential paths.

Sites of attachment of fluorescence labels

Here, we have demonstrated that it is possible to attach fluorescence labels at sites throughout a transmembrane helix with no significant effect on the folding kinetics of the protein into lipid vesicles. The sites on helix D all face out from the protein interior and have been labelled previously with spin labels in mixed detergent/lipid micelles. In contrast to the success of folding all of the labelled helix D mutants into lipid vesicles, as well as a site on helix G (206), it was not possible to fold a site on helix A (11) into vesicles when labelled with bimane. The fact that this labelled mutant L11C-bimane can fold into DMPC/Chaps vesicles shows that a label at this position does not interfere with the protein structure. One possibility is that the label prevents helix A inserting into the vesicle bilayer. Insertion/folding of helix A may be critical to folding bR in vesicles. bR folds co-translationally *in vivo*, probably *via* a translocon apparatus, with helix A emerging into the membrane first.^{55,56} Helix A also forms an independently stable helix *in vitro* in bilayers.⁵⁷ Hence, it has been suggested that folding of helix A, together with some of the other N-terminal helices, could be essential for the later parts of the protein to fold. It is therefore possible that if an attached bimane prevents helix A from inserting correctly to give a transmembrane helix, then the rest of the protein cannot fold. This issue may not occur with the later helices, as with helix A already in the bilayer it can aid the insertion and folding of the later helices to give a more stable helical core in the bilayer. This could stabilise the bimane-labelled helices D and G, thus enabling them to insert into the bilayer.

Membrane protein folding

Only subtle changes are observed in the kinetics of a label reporting on the behaviour of a specific site in the protein, as compared to the behaviour of the protein as a whole. This seems to reflect both the cooperativity of the bR folding reaction together with the complex nature of the reaction under study; in that it involves a multi-spanning protein and a two component (SDS and lipid) solvent environment. Nevertheless, the extensive background data on bR folding and the fact that it has been possible to label specific sites along one face of

a helix, enable significant changes in kinetics to be resolved. The model emerging for folding involves the cooperative packing, insertion and folding of helices, with much helix formation occurring early (or already present in SDS), such that helix orientation and packing dominates the stages of the reaction studied here, together with some insertion and formation of the remaining helical structure. A two-stage model has been proposed to account for the thermodynamics of helical membrane protein folding.⁵⁸ The first step involves formation of stable transmembrane helices and the second packing of these helices. A third step could also follow when cofactors bind and structure outside the membrane forms.⁵⁹ One aspect of the two-stage model that is reflected in our kinetic data is the notion of some stable helices. In our case, we suggest that some critical core is necessary, probably involving helix A. Such a core helical structure may in fact already be present in SDS, but the work here indicates that early insertion of helix A seem to be important if the rest of the protein is to fold correctly.

This study shows that it is possible to gain further detail on the mechanism of membrane protein folding from time-resolved site-specific labelling studies. This work adds to the methods that can be used to probe membrane protein folding mechanisms *in vitro*. It extends the use of site-specific labelling methods that have been applied very successfully to studies of protein function and helix movements.^{2,4,36,40,60,61} The site-directed labelling approach complements another membrane protein folding method to target the behaviour of a specific region of the protein. The movement of single Trp mutants of a β barrel protein, OmpA, have been investigated by time-resolving fluorescence quenching of the Trp fluorescence by Br atoms, which were located at different depths in the bilayer through attachment to the lipid chains.⁶²

Materials and Methods

Materials

All phospholipids were obtained from Avanti Phospholipids (Alabaster, AL), monochlorobimane (bimane) was from Molecular Probes and all-*trans*-retinal and SDS (electrophoresis grade) were from Sigma. All other chemicals and reagents were of analytical grade. 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (Chaps) was from Calbiochem.

Mixed DMPC/Chaps micelles and DOPC or L- α -1,2-dipalmitoleoylphosphatidylcholine (DPOPC) vesicles were prepared in 50 mM sodium phosphate buffer (pH 6) as described.^{17,27} Unilamellar lipid vesicles of 50 nm diameter were prepared by extrusion, stored at 25 °C and used within 20 h of extrusion.

All procedures and measurements on bacteriorhodopsin were performed at 25(±0.5) °C, and those involving bimane-labelled protein or retinal were performed in dim red light.

Protein preparation

Mutant proteins G113C, G116C, G120C, V124C and G125C were a kind gift from S. Subramaniam (NIH, Bethesda, MD) and were originally prepared by members of H.G. Khorana's laboratory (MIT, MA), by over-expression of mutated synthetic bO gene in *Escherichia coli* followed by solvent extraction of purple membrane, as described.¹⁶ Mutant proteins I11C and L206C were prepared according to this method. (A third mutant was attempted, V210C on helix G, but for some unknown reason this mutant protein could not be purified.) Briefly, mutagenesis of the bO gene was carried out by restriction fragment replacement in the synthetic bO genes cloned in the vector pSBO2, and plasmids transformed into *E. coli* DH1 strain, with resistance to kanamycin and ampicillin. Cells were grown in 2×YT medium at 30 °C and protein expression was induced by heat shock to 42 °C. Cells were harvested, subjected to a freeze-thaw cycle, and resuspended in 10 mM Tris-HCl (pH 8.0) containing protease inhibitor (Complete Protease Inhibitor Tablet; Roche). Following the addition of MgCl₂ (to 1 mM), DNase and RNase (to 10 µg/ml) the suspension was rotated at room temperature (RT) for 10 min, then transferred to ice for 10 min. To achieve maximum lysis, cells were disrupted by a single passage through a French pressure cell at 1000 psi (1 psi ≈ 6.9 kPa). The membrane fraction was obtained by ultracentrifugation as described,¹⁶ and the pellet was frozen in liquid N₂ and stored at -80 °C. Mutant bOs were extracted from membrane fractions using organic solvent phase separations in chloroform and methanol (including dithiothreitol, DTT). The proteins were purified using ion-exchange in organic solvent on DEAE-Sepharose CL. The protein fractions were subjected to SDS-PAGE analysis, and samples containing bO were pooled and phase-separated by the addition of water followed by and centrifugation. The interface protein pellet was collected, dried and solubilised in chloroform/methanol/triethyl acetate (100:100:1, by vol.) in the absence of DTT. SDS was added as described and samples were stored at a 5:1 SDS to protein mass ratio after evaporating the solvent using a GyroVap GT (Howe) speed vacuum dryer.⁴⁶ The protein was then snap-frozen in liquid N₂ and stored at -20 °C. Wild-type protein was also prepared by over-expression in *E. coli* (referred to as ebO) in the same manner. All procedures using organic solvents were performed using Teflon centrifuge tubes, chlorinated solvent-resistant tubing and glassware.

Labelling of bacteriorhodopsin cysteine mutants

The cysteine mutants were labelled with bimane following a procedure that was based on previous methods,^{34,41} but optimised to ensure maximum bimane binding to bacterio-opsin. bO cysteine mutants (2 mg ml⁻¹ with 5:1 SDS to protein mass ratio) were solubilised in 1 ml of 0.1 M sodium phosphate (pH 6.7), 1% (w/v) SDS, 6 M urea, 10 mM EDTA and this mixture was incubated for 24 h at 37 °C (shorter incubation times were sufficient for some mutants, but 24 h was found to maximise binding for L206C, for example). Bimane was added (to 1 mM) and incubated for 24 h in the dark at room temperature. The reaction was quenched with 20 mM L-cysteine (or glutathione) and dialysed, to remove excess bimane, for two days against four changes of 2 l of 10 mM sodium phosphate (pH 6), 0.2% (w/v) SDS.

Binding of bimane to the bO mutants was determined by SDS-PAGE chromatography, fluorescence and absorption spectroscopy. Free, unbound bimane is only very weakly fluorescent, with the fluorescence at 470 nm increasing greatly upon protein conjugation.⁵⁰ SDS-PAGE analysis was performed using a non-reducing loading buffer and the resulting, unstained gel was visualized under UV light on a transilluminator, which indicated whether any bimane had bound. A fluorescence spectrum of the labelled proteins revealed a fluorescence band centred at 470 nm that also indicated the covalent binding of bimane. The degree of labelling was quantified by UV/visible light spectroscopy and an example absorption spectrum is shown in Figure 8. The molar concentration of bO and of covalently bound bimane were calculated using their extinction coefficients at 280 nm (66,000 M⁻¹ cm⁻¹)⁸ and at 384 nm (6000 M⁻¹ cm⁻¹; Molecular Probes), respectively. The following bimane to protein stoichiometries were determined: 0.3:1 for G113C, 0.3:1 for G116C, 0.4:1 for G120C, 0.4:1 for V124C, 0.5:1 for G125C and 0.6:1 for L206C. Although stoichiometries of between 0.6 and 0.9:1 were sometimes obtained, stoichiometries achievable for bimane labelling were more reproducibly about 0.3 to 0.6:1 (especially for membrane-embedded sites 113, 116 and 120 on helix D). Separation of the labelled and unlabelled protein was unfeasible with the amounts and volumes of proteins used, without a significant scaling up of all preparation procedures. Since only the bimane-labelled protein contributes to the kinetics determined from the bimane fluorescence, this does not affect the major results reported here. However, the folding yields and intrinsic protein fluorescence data on these bimane-labelled mutants will have contributions from the proportion of unlabelled protein present in the samples, since both unlabelled and labelled protein folds. Hence, most comparisons are made between bimane-labelled proteins from bimane data or with unlabelled Cys

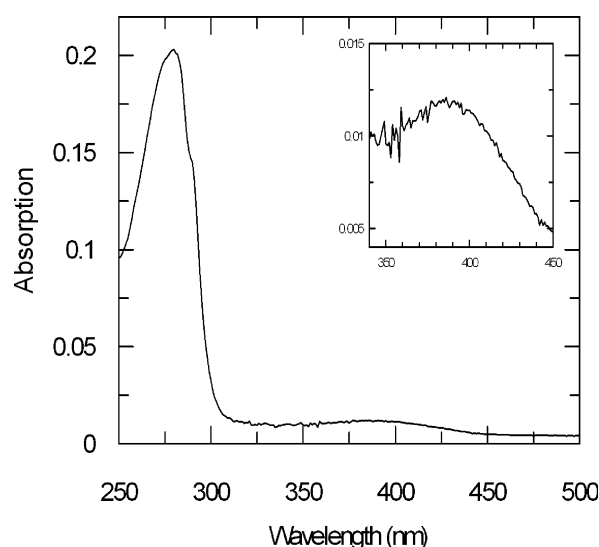


Figure 8. Absorption spectrum for L206C-bimane, showing the protein aromatic side-chain absorption at about 280 nm and that of bimane at about 385 nm (the y axis for this band is expanded in the inset), giving a labelling stoichiometry in this particular case of 0.66:1, bimane per protein. (Note the extinction coefficient of bimane of 6000 M⁻¹ cm⁻¹ is much smaller than that of bO at 280 nm of 66,000 M⁻¹ cm⁻¹.)

mutants. In order to take into account the differences in labelling stoichiometry, all data from bimane fluorescence were normalized both to protein concentration and to the same bimane:protein stoichiometry.

Determination of bR cysteine mutant folding yields

bR was generated from the mutant bO proteins as described.^{11,17,46} The folding to bR was initiated by mixing an unlabelled or labelled bO cysteine mutant (in 0.2% (w/v) SDS) with an equal volume of 2% (w/v) lipid micelles or vesicles, containing all-*trans* retinal (added from ethanol stock, final ethanol concentration <0.5%, v/v) in a ratio of 1:1 protein to retinal. Spectra were recorded from 250 nm to 700 nm and the concentration of folded bR in DMPC/Chaps micelles was assessed by directly measuring its characteristic absorption band centred at about 555 nm, using an extinction coefficient of $55,000 \text{ cm}^{-1} \text{ M}^{-1}$.⁴⁶ As reported previously, such a direct measure is impossible for bR vesicles samples due to high background light-scattering and, in this case, the area under the 555 nm band is determined and converted to a percentage yield, by comparison with the area of the chromophore band for bR folding in a DMPC/Chaps micelles (for the same protein preparation and the same concentration of protein as the vesicle sample).^{17,27,48} The band areas were determined by fitting spectra from 305–700 nm to Gaussian and Rayleigh scattering functions, as described.¹⁷

Steady-state absorption spectroscopy

Absorption spectra were collected using a Varian Cary 1G UV/visible light spectrophotometer with a 2 nm bandwidth and 1 cm path-length. Spectra from lipid vesicle samples were collected with a 1 mm cell path-length, using an integrating sphere accessory and with air as the reference, as described.¹⁷

Steady-state and time-resolved fluorescence measurements

Steady-state fluorescence data were collected using a Fluoromax 2 instrument (Jobin Yvon). Bimane fluorescence spectra were collected between 405 nm and 550 nm, using 1 nm excitation and 10 nm emission bandwidths, with excitation at 375 nm or 384 nm.

Time-resolved fluorescence measurements were performed as described,^{27,46} using an Applied Photophysics SX.18MV stopped-flow spectrophotometer, with a dead-time of approximately 1.4 ms. Labelled mutants (11–16 μM) in 50 mM sodium phosphate (pH 6.0), 0.2% (w/v) SDS were mixed with an equal volume of 2% (w/v) DPOPC or DOPC lipid vesicles in 50 mM sodium phosphate (pH 6.0). The stop volume was 160 μl and the path-length 10 mm. Final concentrations in the stopped-flow cuvette were between 5.5 μM and 8 μM protein, 1% (w/v) DPOPC or DOPC and 0.1% (w/v) SDS. No significance difference was found in the kinetic parameters for folding into DOPC or DPOPC vesicles, values quoted here are for ebO in DPOPC.²⁷ helix D mutants in DOPC and L206C in DPOPC, as these are the most complete data sets in each case, and give the most precise kinetic parameters. Protein fluorescence was excited at 295 nm (1 nm bandwidth, 10 mm path-length) and emission collected above 305 nm, or between 305 nm and 420 nm (for bimane-labelled mutants) using appropriate long band-pass filters. Bimane fluorescence was

measured using an excitation wavelength of 384 nm, and emitted wavelengths were collected above 420 nm by using a filter that cuts off all light below 420 nm. Data were collected over a range of timescales ranging from 0.1 s to 2000 s, as described in detail previously.^{11,27,46} Data were collected also over longer timescales using the Fluoromax 2 instrument with stopped-flow attachment (an Applied Photophysics RX2000 rapid kinetics spectrometer accessory), as described.²⁷ The stopped-flow attachment synchronises the starting point and thus allows averaging between data sets. Experimentally determined rate constants were obtained by fitting the fluorescence data to a sum of exponentials using Grafit 5 software (Erithacus), and the quality of the fits was assessed using a reduced χ^2 criterion and plots of residuals. The exponential components that were resolved were checked for consistency between time scales. Amplitudes were normalized to a final protein concentration of 1 μM and, in the case of the bimane data, to the same bimane to protein stoichiometry.

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