Slow α Helix Formation during Folding of a Membrane Protein†

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Received August 28, 1996; Revised Manuscript Received October 21, 1996⊗

ABSTRACT: Very little is known about the folding of proteins within biological membranes. A "two-stage" model has been proposed on thermodynamic grounds for the folding of α helical, integral membrane proteins, the first stage of which involves formation of transmembrane α helices that are proposed to behave as autonomous folding domains. Here, we investigate α helix formation in bacteriorhodopsin and present a time-resolved circular dichroism study of the slow *in vitro* folding of this protein. We show that, although some of the protein's α helices form early, a significant part of the protein's secondary structure appears to form late in the folding process. Over 30 amino acids, equivalent to at least one of bacteriorhodopsin's seven transmembrane segments, slowly fold from disordered structures to α helices with an apparent rate constant of about 0.012 s^{-1} at pH 6 or 0.0077 s^{-1} at pH 8. This is a rate-limiting step in protein folding, which is dependent on the pH and the composition of the lipid bilayer.

How proteins fold from disordered to native structures is of fundamental biological importance. Although there has been much progress toward understanding this process for water soluble proteins, there is little information available for integral membrane proteins, and how these hydrophobic proteins achieve their final three-dimensional structures is largely a matter of conjecture. Some integral membrane proteins can be unfolded and refolded *in vitro* (Huang et al., 1981; Plumley & Schmidt, 1987; Eisele & Rosenbuch, 1990), and refolding kinetics have now been studied for two α helical proteins, bacteriorhodopsin (Booth et al., 1995, 1996) and the major photosynthetic antenna complex of green plants (Booth & Paulsen, 1996), as well as for β barrel proteins OmpA and OmpF (Surrey & Jähnig, 1995; Surrey et al., 1996).

Bacteriorhodopsin is an integral membrane protein that functions as a proton pump in halobacteria and is closely related to the mammalian vision receptor, rhodopsin, which in turn is a member of the large family of G-protein-coupled receptors (Stoeckenius & Bogomolni, 1982; Hargrave, 1991; Oesterhelt et al., 1992; Khorana, 1993). Several features make bacteriorhodopsin a favorable candidate for a study of membrane protein folding (Popot & Engelman, 1990). Its structure has been determined (Henderson et al., 1990), revealing seven transmembrane helices connected by short extramembrane loops together with a retinal chromophore covalently bound within the helical bundle via a Schiff base link to Lys 216. In addition, bacteriorhodopsin has been shown to refold spontaneously to its native state after denaturation (Huang et al., 1981; London & Khorana, 1982). Refolding can be initiated by mixing denatured apoprotein, bacterioopsin, in sodium dodecyl sulfate (SDS)[1] micelles with mixed dimyristoylphosphatidylcholine (DMPC)/CHAPS micelles containing retinal. The refolding kinetics of bacteriorhodopsin in mixed DMPC/CHAPS micelles have been measured by time-resolving changes in protein fluorescence during folding, using stopped flow mixing to initiate folding (Booth et al., 1995, 1996). Several kinetic phases were resolved, leading to the proposal of the following reaction scheme for the regeneration of bacteriorhodopsin from its SDS-denatured state (bO).

$$\mathrm{bO} \rightleftharpoons \mathrm{I_1} \rightleftharpoons \mathrm{I_2} \overset{\mathrm{R}}{\rightleftharpoons} \mathrm{I_R} \rightarrow \mathrm{I_3} \rightarrow \mathrm{bR}$$

This is essentially the simplest sequential reaction scheme that could be invoked. Intermediates I_1 and I_2 are proposed to be partially folded apoprotein intermediates. Formation of both I_1 and I_2 are independent of retinal, with folding to the latter state being rate-limiting (Booth et al., 1996). It also seems that I_2 has a more or less native-like retinal binding pocket. The subsequent steps in regeneration involve noncovalent binding of retinal to I_2 (giving an intermediate I_R) and finally regeneration of the native chromophore through formation of the Schiff base linkage between retinal and Lys 216. Most apoprotein folding is thought to occur in the steps preceding retinal binding (up to I_2), although some tertiary structure formation could occur in the later (retinal binding) stages.

It has been proposed that the intermediate I_2 corresponds to a state with native secondary structure (i.e. equivalent to seven transmembrane α helices) that accumulates when retinal is omitted from the refolding reaction (London & Khorana, 1982; Booth et al., 1995). However the α helical content of the transient regeneration intermediate, I_2, has not been determined. While time-resolved fluorescence studies readily give kinetic and thermodynamic information on

† This work was funded by the Wellcome Trust (Grant 041153/A/94/Z to P.J.B. and S.L.F.), the Royal Society (P.J.B.), and a BBSRC Biomolecular Sciences Programme Grant to Birkbeck College. P.J.B. is Rosenheim Research Fellow of the Royal Society.

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⊗ Abstract published in *Advance ACS Abstracts,* December 15, 1996.

[1] Abbreviations: bO, bacterioopsin; CD, circular dichroism; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DMPC, L-α-1,2-dimyristoylphosphatidylcholine; DHPC, L-α-1,2-dihexanoylphosphatidylcholine; SDS, sodium dodecyl sulfate.

S0006-2960(96)02199-X CCC: $14.00

transient intermediates, they cannot directly give structural information. A technique which can give information on the secondary structure content during the folding process is circular dichroism (CD) spectroscopy. Two factors must be considered in a CD study of a membrane protein. Firstly, solubilizing detergents (such as CHAPS) absorb in the far-UV, obscuring key CD signals associated with protein secondary structure. Secondly, detergent/lipid micelles scatter light, and any difference in the extent of scattering of left- and right-handed circularly polarized light appears as an artifactual differential absorption in the CD spectra (Mao & Wallace, 1984). The latter scattering problem is overcome instrumentally by a detector geometry that allows collection of virtually all of the scattered light (Mao & Wallace, 1984). The problem of CHAPS absorbing in the far-UV can be overcome by using a new lipid-based refolding system we have recently developed (Booth et al., 1997), where the lipid dihexanoylphosphatidylcholine (DHPC) is substituted for CHAPS [see Booth et al. (1997)]. DHPC has a much lower absorbance than CHAPS in the far-UV which allows measurements of CD as far into the UV as 190 nm. Essentially identical refolding kinetics are observed when denatured bacterioopsin in SDS is refolded in both mixed DMPC/DHPC and DMPC/CHAPS micelles, both in the presence and in the absence of retinal (Booth et al., 1997). In addition, as for DMPC/CHAPS micelles (Booth et al., 1996), only one second-order reaction involving retinal and bacterioopsin can be identified during regeneration of bacteriorhodopsin in DMPC/DHPC micelles, when retinal binds to the partially folded intermediate, I_2. The refolding kinetics were also found to be dependent on both pH and DMPC concentration. Notably, formation of I_2 was slowed both by increasing the concentration of DMPC and by increasing the pH.

Here, we present a time-resolved far-UV CD study of bacteriorhodopsin folding in mixed DMPC/DHPC micelles at pH 6 and 8. Since detector geometries in current stopped flow CD instruments do not permit scattered light collection (and in addition, these stopped flow instruments can rarely monitor changes in the far-UV), we have initiated bacteriorhodopsin refolding by manual mixing of denatured bacterioopsin in SDS with renaturing mixed DMPC/DHPC micelles. Although this limits the time resolution to minutes, the ability to slow the formation of I_2 by changes in pH or DMPC concentration facilitates such measurements and allows us to observe the slow formation of α helices.

MATERIALS AND METHODS

Protein Preparation. Denatured bacterioopsin (bO) in SDS was prepared as described previously (Oesterhelt & Stoeckenius, 1974; Braiman et al., 1987; Booth et al., 1996). DMPC and DHPC were obtained from Avanti Phospholipids and SDS (electrophoresis grade) and *all-trans*-retinal from Sigma. Mixed DMPC/DHPC micelles were prepared by stirring DMPC and DHPC in buffer for 1 h followed by sonication in a bath sonicator for 30 min. Micelles were stored at room temperature and used within 24 h. Bacteriorhodopsin regeneration yields were determined as described previously (Huang et al., 1981; London & Khorana, 1982; Booth et al., 1996).

Fluorescence Measurements. Refolding kinetics were measured as described previously (Booth et al., 1995, 1996), using an Applied Photophysics stopped flow fluorometer, with a dead time of about 1.5 ms. Excitation was at 295 nm (1.2 nm bandwidth), and emission was at >305 nm and was collected with a long pass filter. Folding was initiated by mixing equal volumes of bO in SDS with mixed DMPC/DHPC micelles containing retinal. For addition of retinal to the intermediate I_2, bO in SDS was premixed with DMPC/DHPC micelles for 1 h prior to addition of retinal (Booth et al., 1996). Final concentrations in the measuring cuvette were 2 μM bO, 0.67% DMPC, 0.33% DHPC, and 10 mM phosphate buffer. Refolding kinetics were identical for 2 or 4 μM bO.

Circular Dichroism Measurements. CD spectra were recorded on an Aviv 60DS spectropolarimeter, with a detector acceptance angle of >90° at 22 °C using a 1 mm path length cell. For steady state spectra, data points were collected every 0.2 nm, from 190 to 300 nm, and five scans averaged per sample. Concentrations of denatured bO in SDS were 3.8 μM bO in 0.2% SDS and 10 mM phosphate buffer ($OD_{280} \sim 0.24$ for a 10 mm path length). Protein was refolded by mixing equal volumes of bO in SDS with DMPC/DHPC micelles. Final concentrations were 3.8 μM bO, 0.1% SDS, 0.67% DMPC, 0.33% DHPC (w/v), and 10 mM phosphate buffer. To regenerate bacteriorhodopsin, retinal was included in the DMPC/DHPC micelles, to give a final concentration in the measuring cuvette of 8 μM. CD spectra of refolded protein were collected after 1 h at pH 6 or 5 h at pH 8. Data were analyzed using a least-squares algorithm (Mao & Wallace, 1984) with reference data from Chang et al. (1978).

Kinetics were measured at both 224 and 195 nm. Equal volumes of bO in SDS and DMPC/DHPC micelles (final concentrations as above) were mixed by inverting a 1 mm path length mixing cuvette. Changes in CD were time-resolved after a mixing delay of 20 s.

Experimentally determined rate constants were calculated by fitting data to sums of exponentials. Errors are quoted to one standard deviation, for an average of three measurements, each on a different preparation of bO.

RESULTS

Denatured bacterioopsin in SDS was refolded in mixed DMPC/DHPC micelles (2:1 DMPC:DHPC weight ratio) at both pH 6 and 8. Regeneration yields and kinetics were determined as described previously, using absorption spectroscopy and time-resolved fluorescence, respectively (Booth et al., 1995, 1996). There was a small, reversible reduction in the yield of regenerated bacteriorhodopsin, from about 94% at pH 6 to 83% at pH 8. Figure 1 shows the changes in protein fluorescence with time when bacterioopsin is refolded in mixed DMPC/DHPC micelles in the absence of retinal, resulting in the establishment of an equilibrium between bO and the partially folded apoprotein intermediate I_2. The kinetics of formation of the intermediate I_2 were biexponential, with apparent rate constants of 0.11 and 0.013 s^{-1} at pH 6 and 0.033 and 0.0053 s^{-1} at pH 8 (Booth et al., 1997).

CD spectra (Figure 2) indicate that at both pH 6 and 8 the denatured state of bacterioopsin in SDS has an α helical content of 42%, equivalent to almost four transmembrane α helices, while the refolded state is the same both in the presence and in the absence of retinal and has a native α helical content of about 74%, corresponding to seven transmembrane α helices (London & Khorana, 1982).

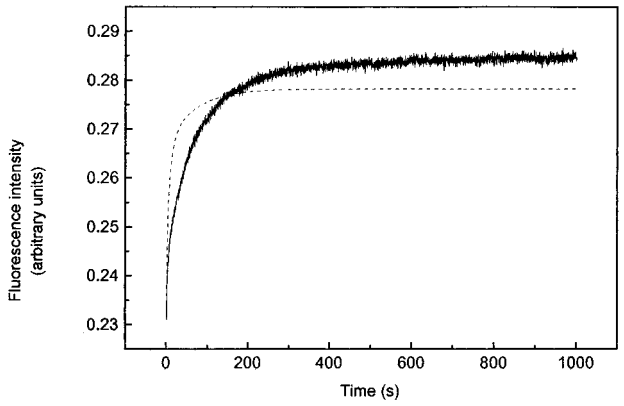

FIGURE 1: Changes in protein fluorescence on folding bO in DMPC/DHPC micelles in the absence of retinal (i.e. formation of intermediate I_2) at pH 8. pH 6 data are also shown for comparison (- - -).

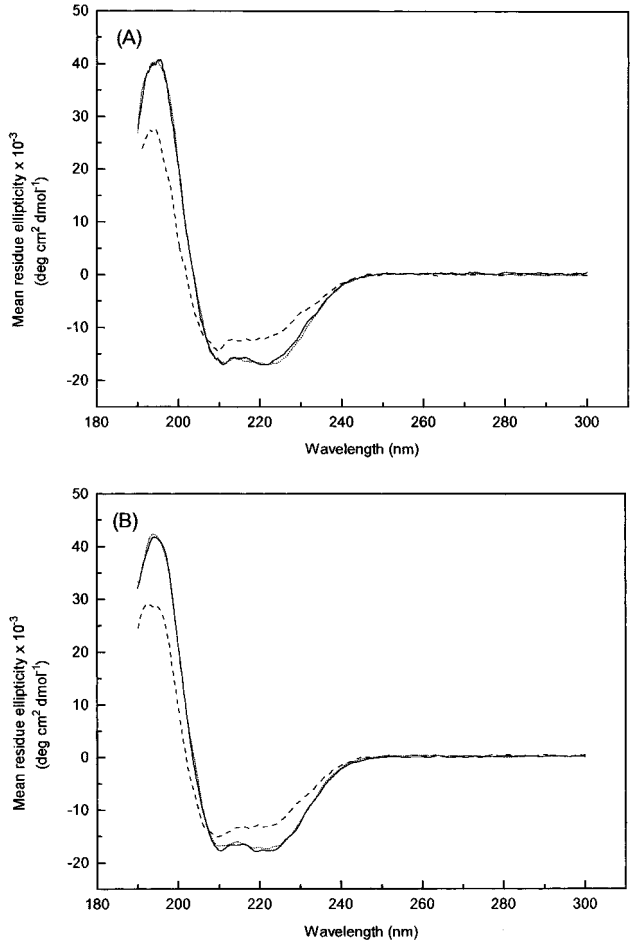

FIGURE 2: CD spectra of denatured bacterioopsin (bO) in SDS, (- - -), and bacteriorhodopsin regenerated in DMPC/DHPC micelles (—) in the absence of retinal and (···), in the presence of retinal: (A) pH 8 and (B) pH 6. Appropriate baselines, collected in the absence of protein, have been subtracted from spectra. Good fits were obtained to data with calculated spectra comprising the following proportions of α helix, random coil, β turn, and β sheet, respectively: (A) 42, 18, 3, and 38% for bO in SDS, 74, 4, 22, and 0% for refolding in the absence of retinal, and 74, 4, 22, and 0% for regenerated bacteriorhodopsin and (B) 43, 18, 6, and 33% for bO in SDS, 74, 4, 22, and 0% for refolding in the absence of retinal, and 78, 1, 21, and 0% for regenerated bacteriorhodopsin.

Figure 3 shows the changes in CD with time at 195 and 224 nm (dead time of 20 s), when bO was refolded in DMPC/DHPC micelles in the presence of retinal to regenerate

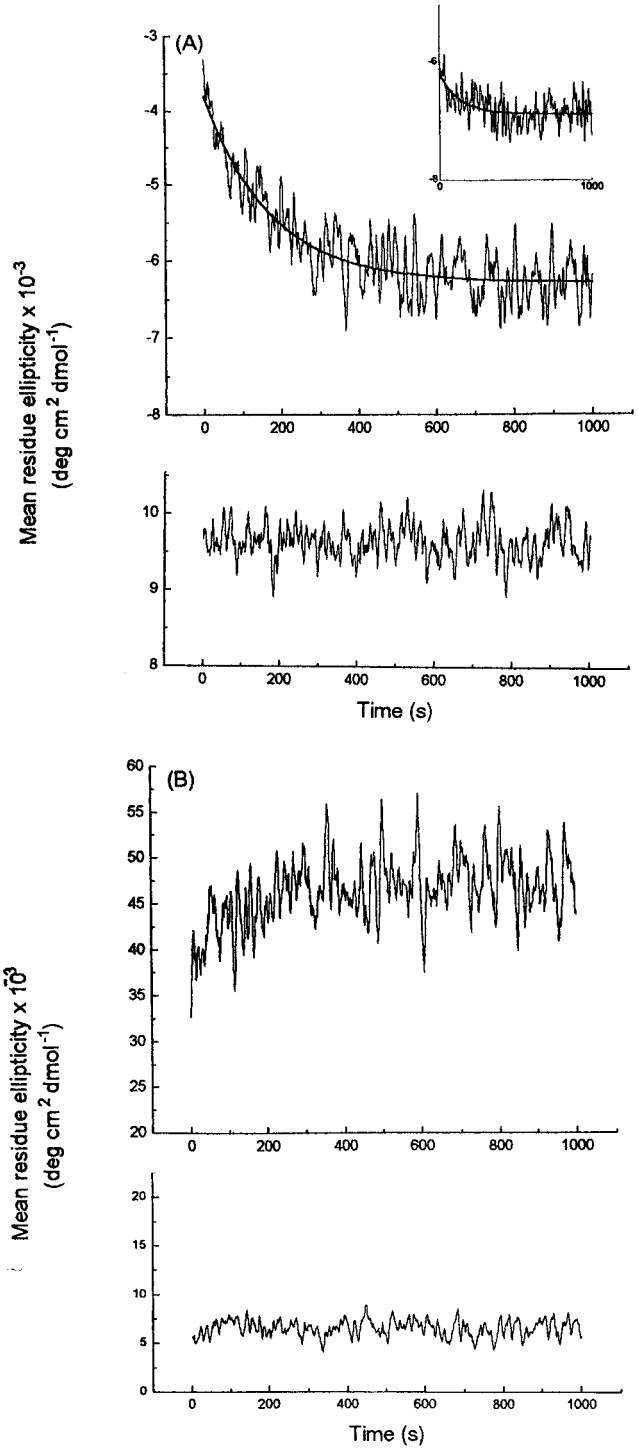

FIGURE 3: Changes in CD at (A) 224 nm and (B) 195 nm, on refolding bO in DMPC/DHPC micelles in the presence of retinal at pH 8. Individual traces are shown, with corresponding baselines below, and with arbitrary *y* axis scales. The inset in panel A shows an example of data collected at pH 6. Each experiment was repeated three times. Monoexponential curve fits are shown in panel A. The mixing dead time was about 20 s, and the zero shown on the *x* axis corresponds to the start of data collection rather than to the start of mixing. Data points were collected over 30 min; only data from the first 1000 s are shown.

bacteriorhodopsin. At both pHs, the increase in positive ellipticity at 195 nm was about 2.5 times that of the increase in negative ellipticity at 224 nm (Figures 2 and 3). Monoexponential kinetics, with the same apparent rate constant, were observed at both wavelengths. Table 1 shows the CD-determined rate constants together with the slower of the

Table 1: Experimentally Determined Rate Constants for Formation of Intermediate I_2

monitoring technique	presence or absence of retinal	rate constant (s^{-1}) pH 6	pH 8
protein fluorescence	absence of retinal	0.013[a]	0.0053[a]
protein fluorescence	presence of retinal	0.012[a]	0.0063[a]
CD at 195 nm	absence and presence of retinal	0.011[b]	0.0072[b]
CD at 224 nm	absence and presence of retinal	0.012[b]	0.0077[c]

[a] From Booth et al. (1997). Only the slower of the two experimentally determined rate constants associated with I_2 formation is given. [b] Rate constant cannot be accurately determined due to the 20 s experimental dead time for the CD measurements, together with the lower signal to noise at 195 nm. [c] Rate constants could be accurately determined at 224 nm at pH 8: 0.0072 ± 0.0022 s^{-1} in the presence of retinal and 0.0077 ± 0.0038 s^{-1} in the absence of retinal.

two rate constants assigned to formation of the intermediate I_2 by fluorescence measurements. Good agreement is observed between the rate constants obtained by both methods. Both fluorescence and CD rate constants are independent of retinal and increase from about 0.012 s^{-1} at pH 6 to about 0.077 s^{-1} at pH 8. In all cases (i.e. at both pH 6 and 8, 195 and 224 nm, and in the presence and in the absence of retinal), the amplitude of the CD-determined component was about 40% of that between the starting state in SDS and the final state in DMPC/DHPC (Figure 2). For example, the amplitude of the kinetic component at 224 nm and pH 8 was 39 ± 11% of that between the start and end states, which corresponds to 31 ± 9 amino acids. The remaining ~60% of the amplitude change between the start and end states occurred within the experimental dead time of 20 s.

DISCUSSION

Bacteriorhodopsin regeneration in DMPC/DHPC micelles occurs according to a reaction scheme in which retinal binds after a rate-limiting step in protein folding to a partially folded apoprotein intermediate, I_2 (Booth et al., 1996). The refolding kinetics associated with formation of intermediate I_2 are slowed by increasing the concentration of DMPC in the mixed DMPC/DHPC micelles, as well as by increasing the pH. This slowing of the kinetics facilitates our time-resolved CD measurements, which have a dead time of about 20 s. The yield of regenerated bacteriorhodopsin also depends on pH, with the optimum yield occurring at pH 6 (London & Khorana, 1982; Booth et al., 1995, 1996). Thus, we have chosen to use DMPC/DHPC micelles where the weight ratio of DMPC to DHPC is 2:1 for the CD measurements reported here, as this allows us to obtain a good estimate of the apparent rate of slow α helix formation at pH 6 (Figure 3 and Table 1). We have also measured the rate of this process at pH 8 where the slower folding allows a more accurate determination of the rate constant.

The use of the DMPC/DHPC refolding system (as opposed to mixed DMPC/CHAPS micelles) allows us to measure changes in CD during bacteriorhodopsin folding at both 224 and 195 nm and thus monitor changes in both α helical and disordered protein structures (Figures 2 and 3). The starting state for our refolding reaction, denatured bacterioopsin in SDS, has an α helical content equivalent to nearly four transmembrane helices, while the end point, both in the presence and in the absence of retinal, has an α helical content equivalent to seven transmembrane helices and a far-UV CD spectrum indicative of native bacteriorhodopsin (London & Khorana, 1982). Thus, in our "refolding" reaction, where denaturing SDS is diluted out by mixed DMPC/DHPC micelles (Huang et al., 1981; London & Khorana, 1982; Booth et al., 1995, 1996), about 70 amino acids form an equivalent of three transmembrane α helices. The relative amplitudes of the CD bands centred on 195 and 224 nm indicate that the amino acids which form these α helices exist in disordered protein structures in SDS-denatured bacterioopsin. Time resolving the changes in CD on refolding bacteriorhodopsin (with a 20 s dead time) reveals monoexponential kinetics that are independent of retinal (Table 1 and Figure 3), with an apparent rate constant of about 0.012 s^{-1} at pH 6 and 0.0077 s^{-1} at pH 8 at both 195 and 224 nm. The relative amplitudes of the CD components indicate that this slow phase represents a conformational change of about 30 amino acids (equivalent to at least one transmembrane α helix) from disordered structures to α helices, resulting in an α helix content equivalent to that of native bacteriorhodopsin. (This slow phase will be referred to as "slow α helix formation".) The other 40 amino acids that form α helices in our refolding reaction have done so within the 20 s experimental dead time.

A comparison of fluorescence- and CD-determined rate constants (Table 1) indicates that the slow α helix formation corresponds to formation of intermediate I_2, which binds retinal. Hence, I_2 contains native secondary structure, as indicated by CD, and the rate-limiting step in I_2 formation reflects folding of at least 30 amino acids to α helices from disordered structures. Folding of bacterioopsin is a multi-exponential process; two experimentally determined rate constants (about 0.11 and 0.013 s^{-1} at pH 6) appear to be associated with I_2 formation, and in addition, there is also a faster component, of about 2 s^{-1} (Booth et al., 1995, 1996). As previously discussed, the simplest sequential scheme for these protein-folding events is one in which the 2 s^{-1} phase represents formation of an intermediate (I_1) which then folds to I_2. However, a branched reaction scheme cannot be excluded as there may, for example, be multiple unfolded forms of the protein (Booth et al., 1995). The time resolution of the CD experiments presented here means that no information can be obtained on the secondary structural changes during these early folding events (2 and 0.11 s^{-1} phases).

CD cannot identify which regions of the protein are involved in the slow α helix formation step. However, the most straightforward interpretation is that disordered structures will probably exist at the membrane surface or in the bilayer interfacial region, where amino acids can hydrogen bond to polar lipid head groups or water, rather than within the interior of the lipid bilayer where they are thermodynamically unfavorable (Lemmon & Engelman, 1994). Since the loops connecting transmembrane α helices in bacteriorhodopsin are short, if the slow phase reflects formation of one complete transmembrane α helix, it is most likely to relate to the N- or C-terminal helix. However, we favor a model in which the core regions of all transmembrane α helices in the hydrophobic bilayer interior form first (they either are already present in our SDS-denatured starting state or form within our experimental dead time). Disordered structures (equivalent to at least 30 amino acids) at the bilayer interfacial region would then later slowly form the ends of some or all of the transmembrane α helices (with a time constant in the minute time range). Although the apparent

rate of this latter α helix formation is slow, the actual intrinsic rate of α helix formation could be much faster, with some other process being rate-limiting, thus making the overall formation of α helices determined by CD appear slow. One possible cause of the apparent slow helix formation is cis−trans isomerization of non-native proline isomers that are present in SDS-denatured bacterioopsin (Booth et al., 1995; Brandts et al., 1975; Nall, 1994). Alternatively, the apparent slow α helix formation could be due to an effect of the lipid bilayer. The apparent rate of helix formation is slowed by increasing the relative proportion of DMPC present in the mixed DMPC/DHPC micelles. An increase in DMPC increases the rigidity to bending of the DMPC/DHPC bilayer (Ben-Shaul et al., 1987) which could result in either a slower rate of insertion of a single transmembrane helix or slower helix association and packing of the core regions of transmembrane α helices in the hydrophobic bilayer interior (Booth et al., 1997). It is possible that in a loosely packed structure the short helix-connecting loops are overstretched and impose strain and a disordered structure on the regions of the protein in the bilayer interfacial region. As a result, the ends of the transmembrane α helices can only form when correct, close packing of the transmembrane α helices is achieved. Thus, as the bending rigidity of the bilayer is increased, slowing the packing of the core regions of the transmembrane helices, the apparent rate of formation of the ends of the transmembrane helices also slows.

A theoretical model has been proposed for the folding of α helical membrane proteins, largely on the basis of thermodynamic arguments and earlier renaturation experiments on bacteriorhodopsin (Popot et al., 1987, 1994; Popot & Engelman, 1990). Folding is simplified to two stages with individual transmembrane helices forming first and then packing in a second stage to give native protein. The latter packing stage is proposed to occur with minimal rearrangement of the preformed α helices so that, although the transmembrane topology remains unchanged, the α helices can bend, tilt, extend, or shorten. Thus, each of the two "stages" is in fact likely to consist of several processes which may lead to the formation of identifiable transient intermediates. In terms of this model, a possible explanation of our data is that only the core regions of transmembrane α helices are formed in stage one. Formation of the intermediate, I_2, involving both extension and packing of these preformed α helices would then occur during stage two.

The slow α helix formation in the minute time range reported here for bacteriorhodopsin is unexpected in view of the much faster formation of α helices in water soluble proteins, where most α helices form in milliseconds or at most a few seconds (Matthews, 1993). Differences are to be expected in the folding of water soluble and membrane proteins. Unlike transmembrane helices, α helices of water soluble proteins are not inherently stable, and while the "hydrophobic effect" seems to speed up events such as α helix formation in the folding of water soluble proteins, the equivalent membrane, lipid−lipid packing effect has more effect on helix association than their formation. Other evidence for slow folding of proteins within membranes comes from studies on the *Escherichia coli* outer membrane protein OmpA. The final step in folding of this membrane protein to its native β barrel structure occurs with a time constant of about 0.5 h (Surrey & Jähnig, 1995).

We report the first observation of α helix formation for a membrane protein. The final stages of α helix formation appear to be slow and rate-limiting in bacterioopsin folding. Our results point toward a role for the lipid solvent in membrane protein folding with bilayer bending rigidity affecting certain folding events. This work, together with the folding studies on β barrel membrane proteins, holds much promise for improving our understanding of how proteins fold within a hydrophobic membrane environment.

ACKNOWLEDGMENT

We thank Dr. Richard Templer for valuable discussions and for suggesting the DMPC/DHPC system. We are also grateful to Amjad Farooq for help with protein preparations, Mike Hanlan for assistance with CD measurements, and Dr. Alison Rodger for helpful comments.

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BI962199R