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Kinetics of an Individual Transmembrane Helix during Bacteriorhodopsin Folding

Emma L. R. Compton, Nicola A. Farmer, Mark Lorch, Jody M. Mason Kathleen M. Moreton and Paula J. Booth*

Department of Biochemistry School of Medical Sciences University of Bristol, Bristol BS8 1TD, UK

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*.

Keywords: membrane protein; folding; kinetics; site-specific label; fluorescence; transmembrane helix

*Corresponding author

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

Present addresses: M. Lorch, Institute for Biophysical Chemistry, Goethe University, Frankfurt, Marie Curie Street 9, 60439 Frankfurt on Main, Germany; J. M. Mason, Institute of Biology III, University of Freiburg, Schaenzlestrasse 1, 79104 Freiburg, Germany.

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.

E-mail address of the corresponding author: paula.booth@bristol.ac.uk

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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

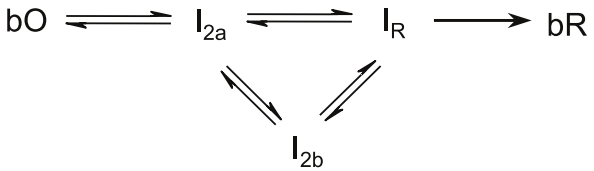

Figure 1. A reaction scheme for folding SDS-bO to bR in lipid vesicles.[27] bO is the SDS-denatured bO starting state that has just over half the native helix content, while bR is the correctly folded state with retinal covalently bound. The central aspects of the scheme are the two I_2 states, I_{2a} 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 $k_{obs}3$), more than one I_R state[63] 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.)

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