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Intermediates in the folding of the membrane protein bacteriorhodopsin

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Assembly of proteins within lipid bilayers is essential for the biogenesis and function of biological membranes. Little is known, however, about the underlying mechanism of assembly, and it is not clear whether it is possible to observe individual folding steps for integral membrane proteins either *in vivo* or *in vitro*. Fluorescence spectroscopy is used here to follow the time course of folding events for bacteriorhodopsin in mixed detergent/lipid micelles. Transient folding-intermediates are detected and binding of the retinal chromophore occurs at a late stage, when it binds to an apoprotein intermediate.

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Some integral membrane proteins can be refolded from a fully denatured state into a functional, native protein. This native state can be achieved spontaneously *in vitro* without the need for accessory proteins. The membrane proton-pump, bacteriorhodopsin from *Halobacteria salinarium* provided the first illustration of this[1,2], and now such refolding has been shown for other membrane proteins, including porins and photosynthetic antennae complexes[3,4].

Bacteriorhodopsin is attractive for studying membrane-protein folding dynamics being one of the few membrane proteins whose native structure is known to near atomic resolution[5]. It has a characteristic seven helical structural-motif and binds a retinal chromophore through a Schiff-base link to a lysine residue. Bacteriorhodopsin can also be efficiently refolded from a denatured state to regenerate functional protein[1,2]. Regeneration efficiencies are easily determined by the extent of recovery of the characteristic purple absorption band of the bound retinal chromophore[1,2], with recovery of this band indicating refolding to a functional, native-like state. Bacteriorhodopsin is part of a family of retinal-containing, integral membrane proteins which also includes the visual receptor rhodopsin, although no sequence homology exists between rhodopsin and bacteriorhodopsin. Rhodopsin, together with other G-protein coupled receptors, may however contain the general motif of seven transmembrane helices[6].

Studies of bacteriorhodopsin regeneration have indicated that denatured apoprotein, bacterio-opsin (bO), can be refolded in mixed detergent/lipid micelles, in the absence of retinal, to form a state, bO', which has native secondary structure[1,2]. Functional bacteriorhodopsin can be regenerated from this state bO' by the addition of retinal and regeneration, observed as the appearance of the purple absorption band which occurs on a time scale of minutes. These observations raise the question as to whether the bO', accumulates in the absence of retinal, is actually an intermediate on the regeneration pathway of bacteriorhodopsin from bO, and whether it is possible to observe such a transient intermediate during folding. A folded apoprotein state has also been observed on bleaching purple membrane[7–9]. Binding of retinal to this bleached membrane was again followed by absorption spectroscopy and, although no kinetics were measured, retinal appeared to bind in two stages. Other work has shown that fragments of bacterio-opsin cleaved in extrinsic loops can also regain native secondary structure in the absence of retinal when incorporated into mixed detergent/lipid micelles[10] or lipid vesicles[11]. On the basis of these experiments a generalised model for membrane protein folding has been proposed[12,13]. This model postulates that folding results from packing of preformed transmembrane helices, each of which represents an autonomous folding domain.

Here, we present a study of the refolding kinetics of bacteriorhodopsin on the millisecond to second time scale. We show that folding of bacteriorhodopsin can be initiated by a rapid-mixing, stopped-flow method, and that changes in protein fluorescence follow the folding and assembly pathway. Taking advantage of the fact that substantial secondary structure formation can occur in the absence of the retinal chromophore[2], we have isolated distinct kinetic stages of bacteriorhodopsin regen-

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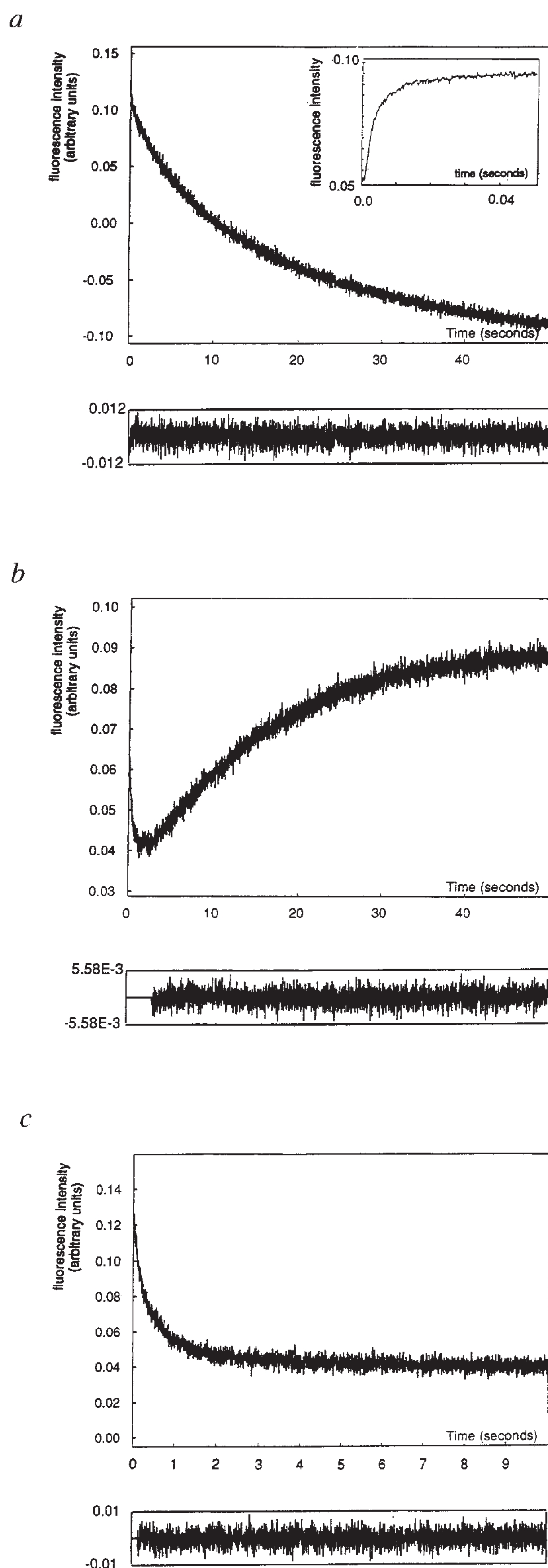

eration, and identified probable intermediates on the regeneration pathway which do not solely reflect chromophore binding. We provide the first evidence that a state analogous to bO' is an intermediate in the regeneration of bacteriorhodopsin. Our results allow us to propose a possible sequential folding scheme for bacteriorhodopsin.

Bacteriorhodopsin folding kinetics

An appropriate starting state for a kinetic study of bacteriorhodopsin folding is denatured apo-protein, bacterio-opsin (bO), in SDS[2]. In this state ~ 60% of the native helical content is present, but the protein is unable to bind its retinal chromophore. Refolding to the native state occurs upon transfer to mixed detergent/lipid micelles (SDS/DMPC/CHAPS) in the presence of retinal[1,2,10,14], with regeneration efficiencies of ~ 80–90% and overall renaturation times of several minutes[1,2]. We have measured the kinetics of this overall refolding of bacterio-opsin by monitoring the accompanying changes in protein fluorescence (Fig. 1*a*). Several kinetic phases are apparent (Table 1): a fast rise in fluorescence (time constant 4 ms, inset Fig. 1*a*), followed by a multiphasic decay (time constants 810 ms and 15 s). In addition there are two longer phases (time constants 50 s and 700 s) which are also seen as increases in absorption, at about 560 nm (not shown), and have already been assigned to Schiff-base chromophore generation as retinal is covalently linked to Lys 216[1,2,15–18].

Apoprotein intermediates

We have regenerated bacteriorhodopsin in two stages: first, folding bO in DMPC/CHAPS micelles in the absence of retinal, to form a state I_O (bO $\rightarrow$ I_O, Fig. 1*b*), and second, by addition of retinal to I_O, to form functional bacteriorhodopsin (I_O $\rightarrow$ bR, Fig. 1*c*). Circular dichroism has already shown that I_O possesses native secondary structure[2]. Omitting retinal from the refolding

Fig. 1 Refolding of bO monitored by changes in protein fluorescence. *a*, overall refolding (bO$\rightarrow$bR): bO refolding in DMPC/CHAPS micelles in the presence of retinal (average of 8 transients). Inset shows data taken on a shorter time scale to resolve the initial, 4 ms, rise in fluorescence (average of 64 transients). Residuals are shown below for a three-exponential fit to the data to resolve time constants τ_1 and τ_2, with the third exponential reflecting the contribution of τ_4 over this time scale. A two-exponential fit gave similar residuals. *b*, bO$\rightarrow I_O$ stage of folding: bO refolding in DMPC/CHAPS micelles in the absence of retinal (average of 32 transients). Residuals are shown for a mono-exponential fit to the data to resolve τ_2 over the range shown. *c*, $I_O \rightarrow$ bR stage of folding: addition of retinal to I_O (average of 4 transients). Note different time scale of *(c)*, to *(a)* and *(b)*. Residuals are shown for a two-exponential fit to the data to resolve τ_3, with the second exponential reflecting the contribution of τ_4 over this time scale. The decay in *(c)* is dominated by a 500 ms component (formation of the Schiff-base which occurs is not shown). Addition of retinol, instead of retinal, resulted in an essentially identical decay at early times (1s component), but no Schiff-base formation was observed.

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Table 1 Fluorescence components resolved on refolding bacterio-opsin

fluorescence component	initial rise[1] time constant/s	τ_1[2] time constant/s	τ_2[2] time constant/s	τ_3[2] time constant/s	τ_4[3] time constant/s
overall refolding bO → bR	0.004 (↑)	0.81 (↓)	15 (↓)	*	50 (↓) 700 (↓)
bO → I_O	0.004 (↑)	0.49 (↓)	15 (↑)	*	*
I_O → bR	*	*	*	0.50 (↓)	70 (↓) 770 (↓)
proposed origin of fluorescence component	micelle mixing	formation of apoprotein intermediate	formation of apoprotein intermediate	non-covalent retinal binding	Schiff-base formation

[1]The initial, 4 ms, rise is not limited by the time resolution of the stopped-flow fluorimeter, but corresponds to mixing of SDS and DMPC/CHAPS micelles. The fluorescence of tryptophan, or fluorescence dyes *N*-phenyl-1-napthylamine (NPN) or Nile Red, increases when 0.2% SDS micelles containing the fluorophore are mixed with DMPC/CHAPS micelles, with the increase having a time constant of 3 ms, 5 ms or 8 ms for 15 μM tryptophan, 5 μM NPN or 50 μM Nile red, respectively. Identical fast fluorescence rises were observed when bO was included in the SDS micelles together with Nile Red.
[2]Values shown for τ_2 are the average of three different samples from the same bO preparation (error to one standard deviation: ±3s). The time constants for τ_2 and τ_3 varied by ~ ±20% for different preparations of bO. The corresponding amplitudes varied by ~ ±15%. The most variability between bO preparations was found for τ_1: the time constant varied by ~ ±30%, and the amplitude by ±60%.
[3]Time constants resolved for τ_4 may be artificially shortened due to the time scale of data collection. The time constants and amplitudes varied by ~ ±25% between bO preparations.

(↓) and (↑) indicate the time constant corresponds to a decay or rise in fluorescence, respectively.

* indicates the kinetic component could not be detected.

process has little effect on the early fluorescence kinetics, indicating that retinal binding is not the first event occurring in regeneration of bacteriorhodopsin. Whether retinal is present or not during refolding an initial, 4 ms, rise in fluorescence is observed which is followed by a decay (τ_1) of hundreds of milliseconds (Table 1). The initial rise in fluorescence almost certainly corresponds to mixing of SDS and DMPC/CHAPS micelles (Table 1). Studies of water soluble proteins suggest that most α-helix formation occurs in less than 2 ms[19–23]. Hence, formation of bacteriorhodopsin transbilayer α-helices might occur during the initial (4 ms) rise in fluorescence, either along with, or prior to, incorporation of the protein into mixed DMPC/CHAPS/SDS micelles. τ_1 is then the first 'folding' event which we can observe, and may represent the formation of an apoprotein folding intermediate, I_1, prior to formation of I_O. Time constants of similar magnitude to τ_1 (tens to hundreds of milliseconds) are also observed during folding of water soluble, mixed α–β proteins where they correspond to stabilisation of secondary structure[20–25].

The final phase of I_O formation, τ_2, is relatively slow (time constant 15 s), and presumably corresponds to the process, $I_1 \rightarrow I_O$. This phase is influenced by retinal (a decay in fluorescence when retinal is present, a rise when retinal is absent). One possible explanation for this kinetic phase is that it corresponds to *cis-trans* isomerisations[26] of the three transmembrane prolines in bacteriorhodopsin[5]. Time constants of 10–100s are typical for *cis-trans* isomerisations in short peptides and water soluble proteins at room temperature[26]. Alternatively, τ_2 could reflect rearrangements of secondary structure elements until a more native-like retinal-binding pocket has formed.

Retinal binding

Retinal binding is accompanied by a quenching of protein fluorescence, presumably due to interactions of retinal with the four tryptophan residues in close contact with the chromophore[5,27]. Binding of retinal to I_O results in a fluorescence decay which is dominated by a 500 ms component (τ_3), with later decay components corresponding to Schiff-base formation (Fig. 1*c*, Table 1). Addition of retinol (an analogue of retinal) to I_O also results in a similar decay component, τ_3 (time constant, 1s). Since retinol can bind non-covalently to bacteriorhodopsin in the retinal-binding site[28] but cannot undergo subsequent Schiff-base formation, this decay component, τ_3, is assigned to formation of a non-covalent intermediate, I_R (I_O and retinal, or retinol). A non-covalent protein-retinal intermediate has also been reported in reconstitution studies of *H. salinarium* apomembrane[8].

The data presented here support a model where I_O is an intermediate on the folding pathway of bacteriorhodopsin, with binding of retinal following I_O formation (bO → I_O → bR). Non-covalent retinal binding is fast compared to the slowest phase of I_O formation (τ_3 is much faster than τ_2). Hence, when retinal is included in the folding reaction from the start, it binds

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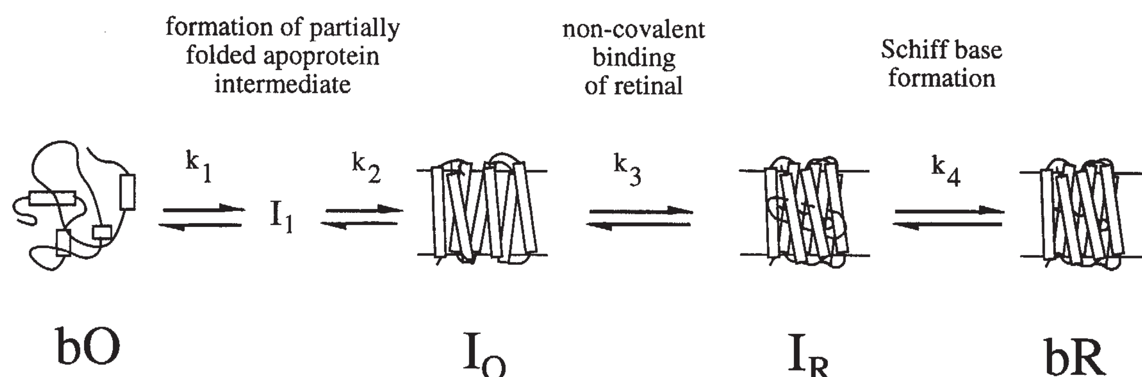

Fig. 2 Tentative scheme for the folding and regeneration of bacteriorhodopsin from a denatured state, bO, in SDS. Only the simplest, sequential folding scheme supported by our data is shown. Following formation of I_O retinal binds in two stages: firstly through noncovalent interactions and finally through a covalent Schiff-base link to Lys 216, to regenerate functional bacteriorhodopsin, bR. All equilibria are assumed to lie to the right hand side and have negligible back reactions. Rate constants (*k*) do not necessarily correspond to the equivalently numbered fluorescence time constants in Table 1, but approximate to this in the simplest case.

non-covalently as soon as I_O forms, and as a result quenches protein fluorescence. This means that the actual non-covalent binding of retinal cannot be resolved in the overall folding reaction and the fluorescence time constant τ_2, which corresponds to I_O formation, is seen as a decay when retinal is present (Table 1). So, although retinal is present in the micelles throughout formation of intermediate I_O it is unable to bind, or indeed quench protein fluorescence, until I_O formation is complete.

The simplest sequential pathway we can propose for regeneration of functional bacteriorhodopsin from a denatured state is shown in Fig. 2. A branched pathway cannot be excluded and there may be multiple unfolded forms of the protein[19], for example arising from proline isomerisation. While we stress that this is a tentative pathway to explain a complex process, a key finding is that it is possible to identify transient intermediates. It is likely that two apoprotein intermediates, I_1 and I_O, form prior to any retinal binding, and a later intermediate, I_R, forms as retinal binds non-covalently to I_O. We can also discount the possibility that the protein folds around the retinal. The binding of the pigment, retinal, appears to occur at a late stage of the protein folding process, perhaps after much of its binding pocket has formed. Thus, although retinal binding contributes to the protein stability[29], it is unlikely that it initiates the assembly of the helix bundle. This determination of the sequence of events and folding dynamics that lead to assembly of bacteriorhodopsin illustrates the feasibility of time-resolved studies of integral membrane-protein folding.

Methods

Protein preparation. Bacteriorhodopsin was prepared as purple membrane from *H. salinarium* (strain S9) using an established procedure[30]. Bacteriorhodopsin was delipidated and denatured to form bO according to the method of Braiman *et al.*[31]. The extent of regeneration of bacteriorhodopsin from bO was determined as described previously by measurement of the purple absorption band of regenerated protein after overnight dark adaptation at room temperature[1,2]. Regeneration extents were typically 65%–85%.

Refolding experiments. Refolding was initiated for overall refolding (bO → bR) experiments by mixing equal volumes of solution 1 (60 μg ml^{-1} (~2 μM) bO, 0.2% SDS, 10 mM phosphate buffer, pH 6.0.) and solution 2 (2% DMPC, 2% CHAPS (mixed DMPC/CHAPS micelles), 10 mM phosphate buffer pH 6.0, 7 μM all *trans*-retinal); for (bO → I_O) stage of refolding by mixing equal volumes of solution 1 and solution 3 (2% DMPC, 2% CHAPS, 10mM phosphate buffer pH 6.0), and for (I_O → bR) stage of refolding by mixing equal volumes of solution 4(60 μg ml^{-1} bO, 0.2% SDS, 1% DMPC, 1% CHAPS 10 mM phosphate buffer pH 6.0 (pre-equilibrated for 6 hours)) and solution 5 (1% DMPC, 1% CHAPS, 7 μM retinal, 10mM phosphate buffer pH 6.0). To allow for different regeneration extents of different bO preparations, bO concentrations were varied slightly for different preparations. Concentrations were normalised to give the same final chromophore absorbance on regeneration. Mixed DMPC/CHAPS micelles were sonicated using a bath sonicator for 30 minutes. Experiments were done at 20 °C using an Applied Photophysics SX.17MV stopped-flow flurorimeter, deadtime ~1.4 ms. For measurements of protein fluorescence, excitation was at 290 nm (1.2 nm bandwidth) and emission ≥305 nm selected with a long-pass filter. For Nile Red excitation was 560 nm (2.3 nm bandwidth) and emission ≥610 nm, and for NPN excitation was 340 nm (2.3 nm bandwidth), emission ≥420 nm. The pathlength for all measurements was 10 mm.

Data analysis. Time constants are the reciprocals of the experimentally determined rate constants. Rate constants were calculated from fluorescence data by iterative reconvolution based on the Marquardt fitting algorithm, assuming multiexponential kinetics. Time constants were determined from measurements taken on different time scales (2000 or 4000 data points per scale): 20 ms and 50 ms full scale for the initial rise; 2 s and 5 s full scale for τ_1; 50 s and 200 s full scale for τ_2 and 1000 s and 2000 s for τ_4. Where necessary ranges were selected so that data corresponding to rises or decays in fluorescence were analysed separately.

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