



COMMUNICATION

Probing Membrane Protein Unfolding with Pulse Proteolysis

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Technical challenges have greatly impeded the investigation of membrane protein folding and unfolding. To develop a new tool that facilitates the study of membrane proteins, we tested pulse proteolysis as a probe for membrane protein unfolding. Pulse proteolysis is a method to monitor protein folding and unfolding, which exploits the significant difference in proteolytic susceptibility between folded and unfolded proteins. This method requires only a small amount of protein and, in many cases, may be used with unpurified proteins in cell lysates. To evaluate the effectiveness of pulse proteolysis as a probe for membrane protein unfolding, we chose *Halobacterium halobium* bacteriorhodopsin (bR) as a model system. The denaturation of bR in SDS has been investigated extensively by monitoring the change in the absorbance at 560 nm (A_{560}). In this work, we demonstrate that denaturation of bR by SDS results in a significant increase in its susceptibility to proteolysis by subtilisin. When pulse proteolysis was applied to bR incubated in varying concentrations of SDS, the remaining intact protein determined by electrophoresis shows a cooperative transition. The midpoint of the cooperative transition (C_m) shows excellent agreement with that determined by A_{560} . The C_m values determined by pulse proteolysis for M56A and Y57A bRs are also consistent with the measurements made by A_{560} . Our results suggest that pulse proteolysis is a quantitative tool to probe membrane protein unfolding. Combining pulse proteolysis with Western blotting may allow the investigation of membrane protein unfolding *in situ* without overexpression or purification.

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Abbreviations used: bR, *H. halobium* bacteriorhodopsin; CHAPSO, 3-([3-cholamidopropyl]dimethylammonio)-2-hydroxy-1-propanesulfonate; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine.

Introduction

Understanding how the sequence information of a protein encodes its three-dimensional structure, often termed the protein folding problem, is one of the greatest challenges in science. Quantitative assessment of kinetics and thermodynamics of protein folding has been an essential component of the pursuit of this challenge. By investigating kinetics and thermodynamics of protein folding, we have learned a great deal about the mechanistic details of protein folding and the forces stabilizing protein structures. Our understanding of membrane protein folding is, however, significantly underdeveloped relative to that of water-soluble proteins.¹⁻⁵ This disparity is mostly attributable to the technical difficulties of studying membrane proteins.

One of the common challenges that researchers encounter in the investigation of membrane proteins is the difficulty in obtaining pure membrane protein in the quantity necessary for biophysical studies.⁶ Membrane proteins exist in an anisotropic environment composed of both aqueous and lipid solvents. Due to the difficulty in maintaining this complex solvent environment, purified integral membrane proteins tend to behave poorly in biophysical studies.⁷ Another common limitation to such investigations is that the lipids and detergents that are required to maintain folded membrane proteins may interfere with spectroscopic or calorimetric methods.⁸⁻¹⁰ Finally, the denatured states of integral membrane proteins may retain a significant amount of secondary and tertiary structures.^{11,12} Minimal difference in the signal of the native and the denatured species may compromise the ability to monitor folding and unfolding of membrane proteins using common spectroscopic probes.^{13,14} As a result, many experimental methods developed to investigate folding of water-soluble proteins are often not so useful in the investigation of membrane protein folding. Novel conformational probes that may circumvent the current technical difficulties and may complement existing techniques are needed to support the growing interest in membrane protein folding.^{15,16}

To address the need for novel probes to investigate membrane protein folding, we tested the validity of pulse proteolysis as a probe for membrane protein folding. Pulse proteolysis is a method to determine the fraction of native protein (f_N) with a short pulse of proteolysis.^{17,18} Folded and unfolded proteins typically have very distinct proteolytic susceptibility. Thus, one can determine f_N by quantifying the amount of remaining intact protein after unfolded proteins are selectively digested by pulse proteolysis. We have shown that pulse proteolysis is a robust and versatile method to measure thermodynamic stability and unfolding kinetics of soluble proteins.^{17,18} Pulse proteolysis requires much less protein than conventional

approaches. Determination of thermodynamic stability is possible with less than 100 μg of protein. Also, the use of gel electrophoresis for quantification of the remaining intact protein eliminates the need to purify a protein for folding studies. Furthermore, by coupling pulse proteolysis with quantitative Western blotting (Pulse and Western), folding and unfolding of low-abundance proteins can be studied in a cell lysate.¹⁹ These merits of pulse proteolysis may be particularly useful in the investigation of membrane proteins, which are typically difficult to express and purify. Also, because pulse proteolysis does not rely on spectroscopic properties of proteins, the method may be applicable even when unfolding does not result in significant changes in spectroscopic signals or when lipids or detergents interfere with the measurement.

To test the applicability of pulse proteolysis to membrane protein folding studies, we chose *Halo-bacterium halobium* bacteriorhodopsin (bR), a seven-helical transmembrane protein, as a model. The structural integrity of the protein can be monitored easily by the absorbance at 560 nm (A_{560}) because bR has a retinal cofactor. Due to this convenient probe and the relatively easy purification of the protein, bR has been a popular model system for membrane protein folding studies. Moreover, it has been shown that unfolding of bR by SDS is reversible^{13,20} and that the free energy of unfolding (ΔG_{unf}) of bR in the transition zone is linearly proportional to mole fraction of SDS (X_{SDS}) in mixed micelles.^{21,22} This empirical relationship makes bR a valuable system to investigate energetic principles governing thermodynamic stability of membrane proteins. Furthermore, kinetic analysis of bR folding and unfolding in mixed micelles, with varying X_{SDS} , shows that bR demonstrates simple two-state folding behavior.²² This two-state behavior has also led to the characterization of the folding transition state of bR using Φ -value analysis.²³ The great deal of information on the thermodynamic stability and folding/unfolding kinetics of bR makes this protein an ideal model to test the validity of pulse proteolysis as a probe for unfolding of membrane proteins. Here, we report our successful application of pulse proteolysis to monitor the denaturation of bR in a quantitative manner. To assess the accuracy of the method, we compared f_N determined by pulse proteolysis with f_N determined by A_{560} . We also demonstrate that pulse proteolysis is a valid tool to determine the effect of mutations on the membrane protein stability by applying pulse proteolysis to two variants of bR.

Subtilisin is a suitable protease for pulse proteolysis in SDS

It was necessary to ensure that proteolytic activity is maintained in the presence of SDS in order to test the validity of pulse proteolysis as a probe for bR

denaturation in SDS. Pulse proteolysis of soluble proteins typically uses thermolysin to digest unfolded protein in urea.^{17,24} However, we find that thermolysin is rapidly inactivated by SDS (data not shown). This inactivation seems to result from precipitation of SDS and Ca^{2+} , which is an essential metal ion for the structural integrity of thermolysin. We therefore tested subtilisin, another robust bacterial protease with broad specificity. It has been reported that subtilisin retains its structure in the presence of SDS.²⁵

To determine whether subtilisin is active enough for pulse proteolysis in SDS, we evaluated the enzyme activity under the conditions used for the denaturation of bR. Unfolding of bR is typically investigated by titrating bR in lipid bicelles composed of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), a saturated lipid, and of 3-([3-cholamidopropyl]dimethylammonio)-2-hydroxy-1-propanesulfonate (CHAPSO), a non-denaturing detergent, with SDS.²¹ The activity of subtilisin was determined by measuring the rate of the cleavage of a fluorogenic substrate, *o*-aminobenzoyl-Ala-Gly-Leu-Ala-*p*-nitrobenzylamide, by subtilisin in the presence of 15 mM DMPC, 16 mM CHAPSO, and varying concentrations of SDS. The increase in fluorescence from the cleavage of the substrate fit well to a first-order rate equation at all concentrations of SDS tested, which indicates that subtilisin is not inactivated during the assays. Still, our assay shows that the $k_{\text{cat}}/K_{\text{m}}$ value decreases as SDS concentration is increased (Supplementary Fig. S1). The apparent inhibition of subtilisin by SDS has been previously explained by the partitioning of peptide substrates into SDS micelles.²⁵ Nevertheless, subtilisin retains enough catalytic activity for pulse proteolysis even when the mole fraction of SDS in mixed micelles (X_{SDS}) is 0.85, which is significantly higher than the known C_{m} value of bR ($X_{\text{SDS}}=0.72$) under the same condition.²² This result suggests that subtilisin is a suitable protease for pulse proteolysis in the presence of SDS.

Denaturation by SDS significantly increases the proteolytic susceptibility of bacteriorhodopsin

In order for pulse proteolysis to serve as a valid probe for bR unfolding, the SDS-denatured state must be significantly more susceptible to proteolysis than the native state. In the case of bR, it is known that the SDS-denatured species primarily exists within the membrane and retains about 40% α -helical content.^{13,14} To determine whether native and SDS-denatured bRs have distinct proteolytic susceptibility, we measured the rate of proteolysis at $X_{\text{SDS}}=0.60$ and $X_{\text{SDS}}=0.83$, in which bR is native and denatured, respectively.²² The disappearance of intact bR by proteolysis fit well to a first-order rate equation under all conditions. To determine $k_{\text{cat}}/K_{\text{m}}$

value reliably and to learn the nature of the rate-limiting step for proteolysis, we determined the proteolysis rate of bR with different concentrations of subtilisin (Fig. 1). The apparent $k_{\text{cat}}/K_{\text{m}}$ values for SDS-denatured bR ($43,000 \pm 2000 \text{ M}^{-1} \text{ s}^{-1}$) is about 30-fold greater than that for native bR ($1500 \pm 100 \text{ M}^{-1} \text{ s}^{-1}$). This result clearly demonstrates that SDS-denatured state of bR is more proteolytically susceptible than native bR. When estimated with these kinetic constants, $\sim 85\%$ of native bR and less than 1% of SDS-denatured bR would remain intact after 1-min pulse with 50 $\mu\text{g}/\text{mL}$ subtilisin. Also, the linear correlation between the proteolysis rate and the protease concentration suggests that the proteolysis step catalyzed by the enzyme, not the preceding conformational change in bR, is the rate-limiting step for the overall proteolysis.²⁶

Though the sequences of the cleavage sites in bR are different from that of the peptide substrate, the comparison of the $k_{\text{cat}}/K_{\text{m}}$ values for bR with those for the peptide substrate provides valuable insight into the conformations of native and SDS-denatured bRs. At $X_{\text{SDS}}=0.53$, a condition similar to the condition chosen for native bR proteolysis measurements ($X_{\text{SDS}}=0.60$), the $k_{\text{cat}}/K_{\text{m}}$ value of the fluorogenic tetrapeptide substrate is $27,000 \text{ M}^{-1} \text{ s}^{-1}$, which is about 20-fold greater than that of native

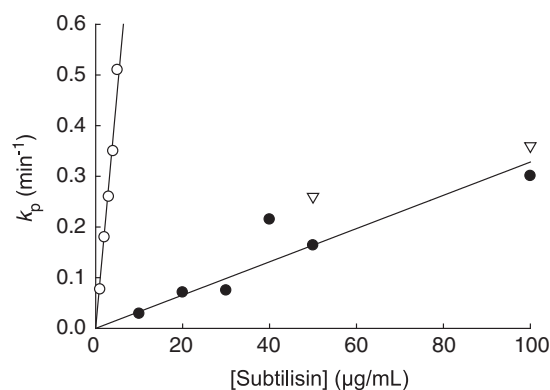


Fig. 1. Kinetics of proteolysis of wild-type bacteriorhodopsin by subtilisin. Kinetics of proteolysis of bR by subtilisin was determined under a native condition at 0.60 X_{SDS} (●), an SDS-denaturing condition at 0.83 X_{SDS} (○), and at 0.75 X_{SDS} , the observed C_{m} by pulse proteolysis (▽). bR was pre-equilibrated in 10 mM sodium phosphate buffer (pH 6.0) containing 15 mM DMPC and 16 mM CHAPSO for at least 1 h. bR was then diluted into the same buffer containing the designated X_{SDS} to a final protein concentration of 0.10 mg/mL and was allowed to equilibrate for 3 min. Following the equilibration, we added varying concentrations of subtilisin to each reaction. Reactions were quenched by the addition of PMSF to the final concentration of 13 mM at designated time points. The kinetic constants (k_p) were determined by fitting the disappearance of intact bR over time on an SDS-PAGE gel to a first-order rate equation. The $k_{\text{cat}}/K_{\text{m}}$ values at 0.60 X_{SDS} and 0.83 X_{SDS} were determined from the slope of the plots.

bR ($1500 \pm 100 \text{ M}^{-1} \text{ s}^{-1}$). Thus, native bR is significantly less susceptible than the peptide substrate. This is presumably either because bR is structured and exists exclusively within mixed micelles or because the sequence of the cleavage site in bR is not as favorable as the peptide substrate for subtilisin. Interestingly, under denaturing conditions ($0.82 X_{\text{SDS}}$), the k_{cat}/K_m value of the peptide substrate is $1400 \text{ M}^{-1} \text{ s}^{-1}$, which is about 30-fold lower than the k_{cat}/K_m value of SDS-denatured bR ($43,000 \pm 2000 \text{ M}^{-1} \text{ s}^{-1}$). When it is considered that bR may have less favorable cleavage sequences than the peptide substrate, the greater susceptibility of SDS-denatured bR than that of the peptide substrate is intriguing. Under this condition, it is likely that the peptide substrate is mostly partitioned into the micelles, but SDS-denatured bR contains solvent-accessible unstructured loops that are easily cleavable by the protease.

The nature of the SDS-denatured state of bR remains a subject of some controversy. The loss of the absorbance at 560 nm (A_{560}) upon denaturation by SDS suggests a drastic change in the microenvironment around the bound retinal. Interpretation of the CD spectra suggested the SDS-denatured state of bR has about 40% less helical content than native bR.¹⁴ However, based on disagreement between α -helical content as determined by CD and NMR for several proteins in the presence of SDS, it has been suggested that there is no sufficient evidence for a loss of secondary structure in the SDS-denatured state of bR.¹⁰ It is noteworthy that the increased proteolytic susceptibility of SDS-denatured bR relative to that of native bR is consistent with the observed loss of structure in the SDS-denatured state. More recently, hydrogen-deuterium exchange and oxidative methionine labeling suggest that SDS-denatured bR contains several unstructured regions,^{11,12} which is also consistent with our observation.

Pulse proteolysis reports a cooperative unfolding transition of bacteriorhodopsin in SDS

The validity of pulse proteolysis as a probe for bR unfolding was assessed in comparison to A_{560} . Pulse proteolysis was performed under the experimental conditions previously designed to monitor bR unfolding by A_{560} .²¹ After 3-min equilibration of bR in solutions with varying X_{SDS} , each sample was incubated with $50 \mu\text{g}/\text{mL}$ subtilisin for 1 min. According to the proteolysis kinetics discussed above, 1-min incubation with this concentration of subtilisin ensures nearly complete digestion of SDS-denatured bR while minimizing the digestion of the native bR. The remaining intact bR in each sample after pulse proteolysis was analyzed by SDS-PAGE (Fig. 2a). The majority of bR remains intact, following pulse proteolysis, under native conditions

($X_{\text{SDS}} < 0.70$), which is consistent with the proteolysis kinetics discussed above. Complete digestion of unfolded bR at $X_{\text{SDS}} = 0.81$ confirms that subtilisin retains sufficient activity in SDS for pulse proteoly-

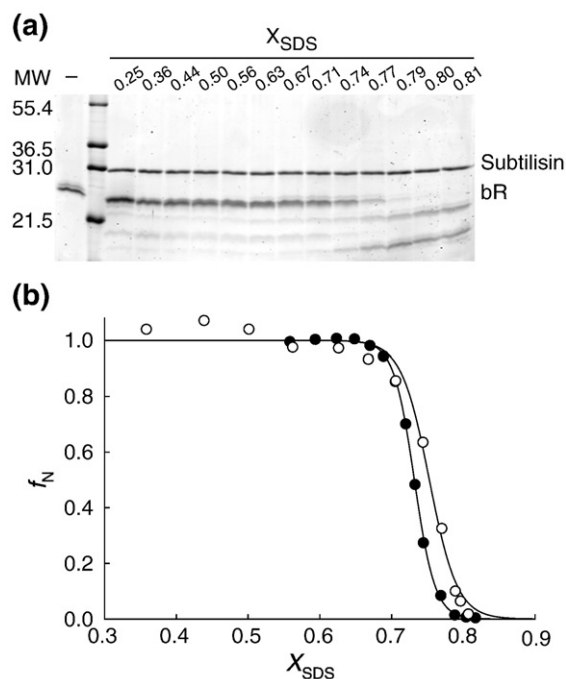


Fig. 2. Pulse proteolysis of wild-type bacteriorhodopsin. (a) A representative SDS-PAGE gel following pulse proteolysis of bR is shown. bR was pre-equilibrated in 10 mM sodium phosphate buffer (pH 6.0) containing 15 mM DMPC and 16 mM CHAPSO for at least 1 h. bR was then diluted into the same buffer containing varying concentrations of SDS. Reactions were incubated for 3 min before the initiation of pulse proteolysis by the addition of subtilisin to $50 \mu\text{g}/\text{mL}$. After 1 min, we quenched reactions by the addition of PMSF to 10 mM. Quenched reactions were then analyzed by SDS-PAGE. Undigested bR (–) is shown for comparison. (b) The f_N values of wild-type bR in SDS were determined by pulse proteolysis (O) and by A_{560} (●). The f_N values were determined from pulse proteolysis by dividing the remaining intact bR intensities by the intensity of the upper baseline (I_0) value derived from the fitting of the data set to the two-state equilibrium unfolding model. Equilibrium unfolding of bR was monitored by A_{560} as previously described.²¹ Briefly, bR was pre-equilibrated in 10 mM sodium phosphate buffer (pH 6.0) containing 15 mM DMPC and 16 mM CHAPSO for at least 1 h. bR was then diluted into the same buffer to a final concentration of 0.10 mg/mL. The protein was titrated with 10 mM sodium phosphate buffer (pH 6.0) containing 20% SDS, 15 mM DMPC, and 16 mM CHAPSO, to raise the X_{SDS} . Following each addition of the solution, we stirred the reaction for 3 min in the dark before the A_{560} value was read. Data were fit to a two-state equilibrium unfolding model. The X_{SDS} at which half of the protein is denatured (C_m) determined by pulse proteolysis and by A_{560} are $0.753 \pm 0.003 X_{\text{SDS}}$ and $0.732 \pm 0.001 X_{\text{SDS}}$, respectively.

sis. Also, proteolysis of native and SDS-denatured bR species show distinct cleavage patterns, producing three discernable cleavage products and two discernable cleavage products, respectively. This difference in cleavage patterns further confirms that bR experiences a significant conformational change upon denaturation by SDS.

The plot of normalized band intensities of intact bR *versus* X_{SDS} shows a cooperative transition (Fig. 2b). The transition midpoint (C_m) value of $0.753 \pm 0.003 X_{\text{SDS}}$ was determined by fitting the plot to a two-state equilibrium unfolding model. The average C_m values of wild-type bR determined in triplicate experiments was $0.749 \pm 0.005 X_{\text{SDS}}$. These results demonstrate that C_m determination by pulse proteolysis has good precision. For comparison, unfolding of bR under the identical condition was also monitored with A_{560} as described previously²¹ (Fig. 2b). The transition monitored by A_{560} is very similar to the transition monitored by pulse proteolysis. The C_m value determined by A_{560} ($0.732 \pm 0.001 X_{\text{SDS}}$) is in good agreement with the C_m value determined by pulse proteolysis. The coincidental change in proteolytic susceptibility and the disruption of the retinal binding site suggests that the denaturation of bR by SDS is cooperative.

A key assumption in the application of pulse proteolysis is that the amount of native protein that is digested during the reaction is negligible.^{17,24} However, the observed rate of proteolysis of native bR at $X_{\text{SDS}}=0.60$ (Fig. 1) indicates that about 15% of folded bR is digested during 1-min pulse with 50 $\mu\text{g}/\text{mL}$ subtilisin. To estimate the experimental error in C_m introduced by the proteolysis of native bR, we repeated pulse proteolysis with higher concentrations of subtilisin (100 and 200 $\mu\text{g}/\text{mL}$). The amount of the remaining intact bR after pulse proteolysis was clearly lower when higher concentrations of subtilisin were used (data not shown). However, the C_m values were not affected significantly; the C_m values determined with 100 and 200 $\mu\text{g}/\text{mL}$ subtilisin were $0.756 \pm 0.003 X_{\text{SDS}}$ and $0.754 \pm 0.008 X_{\text{SDS}}$, respectively. This result demonstrates that C_m determination of wild-type bR is not affected by the proteolysis of native bR. One possible explanation for the independence of the C_m value on the concentration of subtilisin used in the pulse is that similar fractions of native bR are digested at different X_{SDS} . To test this possibility, we measured the proteolysis rate of native bR at $X_{\text{SDS}}=0.75$, the observed C_m value, in the presence of 50 and 100 $\mu\text{g}/\text{mL}$ subtilisin. The observed rate constants at 0.75 X_{SDS} are close to those observed at 0.60 X_{SDS} (Fig. 1). This result suggests that the proteolysis rate of native bR is somewhat independent of X_{SDS} , which is also consistent with the flat upper baseline of the normalized band intensity *versus* X_{SDS} plot (Fig. 2b). The similar proteolysis rates of native bR seem to result in a uniform

decrease in band intensities and consistent C_m values that are independent of protease concentration. Therefore, the proteolysis of native bR during a pulse does not cause any systematic error in C_m determination. It is also notable that SDS does not affect the rate of proteolysis of native bR by subtilisin but does affect that of the peptide substrate. This difference is again consistent with the suggestion that the decrease of subtilisin activity at higher SDS concentration is not from the inhibition of subtilisin by SDS but from the partitioning of soluble substrate into SDS micelles.²⁵ The rate of proteolysis of bR by subtilisin is likely to be independent of SDS concentration simply because bR does not experience a significant change in its distribution between the soluble phase and the mixed micelle phase.

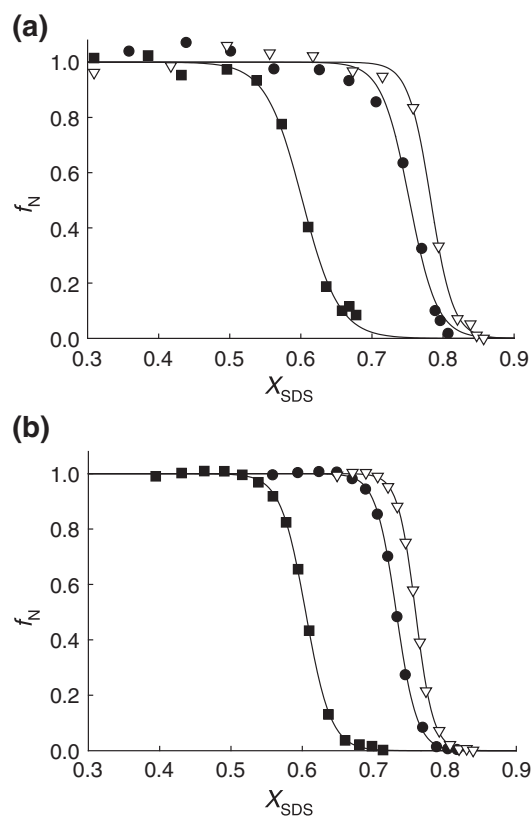


Fig. 3. Effect of mutations on the denaturation of bR by SDS monitored by pulse proteolysis and by A_{560} . Denaturation of wild-type (●), M56A bR (▽), and Y57A bR (■) by SDS was monitored by pulse proteolysis and by A_{560} . (a) Pulse proteolysis was performed for M56A and Y57A bRs as described in Fig. 2. Quenched reactions were then analyzed by SDS-PAGE. The intensity of remaining mutant bR after pulse proteolysis is converted to f_N and is plotted against X_{SDS} for each reaction. (b) The A_{560} values of bR were also converted to f_N and were plotted against X_{SDS} . Reactions were performed as described in Fig. 2. Wild-type bR data are shown for comparison.

Table 1. C_m values of wild-type, M56A, and Y57A bacteriorhodopsins determined by pulse proteolysis and by A_{560}

Bacteriorhodopsin	Pulse proteolysis		A_{560}	
	C_m^a (X_{SDS})	ΔC_m^b (X_{SDS})	C_m^a (X_{SDS})	ΔC_m^b (X_{SDS})
Wild type	0.753 ± 0.003	—	0.732 ± 0.001	—
M56A	0.783 ± 0.002	0.034 ± 0.005	0.759 ± 0.001	0.027 ± 0.001
Y57A	0.602 ± 0.003	-0.147 ± 0.004	0.605 ± 0.001	-0.127 ± 0.001

^a C_m values (± standard error) were determined by fitting the band intensities at different X_{SDS} to a two-state equilibrium unfolding model. C_m values of wild-type bR determined by pulse proteolysis in triplicate measurements have an SD of 0.005 X_{SDS} .
^b $\Delta C_m = C_m$ (mutant) - C_m (wild type).

To demonstrate that pulse proteolysis is capable of detecting changes in membrane protein stability, we characterized M56A and Y57A bRs, which have previously shown to be more stable and less stable variants, respectively, relative to wild-type bR.²¹ Pulse proteolysis of M56A and Y57A bRs was performed in varying X_{SDS} with 50 $\mu\text{g}/\text{mL}$ subtilisin as described above (Fig. 3a). Denaturation of the two variants by SDS was also characterized using A_{560} for comparison (Fig. 3b). The observed C_m values are reported in Table 1. The change in the C_m value relative to wild-type (ΔC_m) by pulse proteolysis are consistent with ΔC_m as determined by A_{560} measurements for both bR point mutants. This result demonstrates that pulse proteolysis is an efficient and reliable tool to monitor the variation in membrane protein stability.

Pulse proteolysis as a probe for membrane protein folding

This work demonstrates that pulse proteolysis is an effective tool to monitor denaturation of a membrane protein in a quantitative manner. Though pulse proteolysis was originally developed to study folding and unfolding of soluble proteins in urea, this simple but quantitative method seems to be particularly useful in the investigation of membrane proteins. We have recently shown that the stability of low-abundance proteins can be determined in a cell lysate by combining pulse proteolysis with quantitative Western blotting.¹⁹ Our successful application of pulse proteolysis to bR suggests that, if an antibody is available for a membrane protein, folding and unfolding of that protein may be investigated by this approach without overexpression or purification.

Additionally, it is known that, in several cases, the denatured state of a membrane protein may contain a significant amount of structure that may complicate spectroscopic differentiation of native and denatured species.^{13,14,27} In this work, we have demonstrated that proteolysis is a sensitive probe that can distinguish between the native and the denatured states of a membrane protein, regardless of their optical properties. It is also noteworthy that CHAPSO is known to complicate the use of far-UV

CD as a probe because of the absorbance of its amide group.⁹ We show here that pulse proteolysis is not prohibited by the presence of DMPC, CHAPSO, and up to 5% (w/v) SDS. Thus, pulse proteolysis must be useful in the investigation of the folding of various membrane proteins that are challenging to be studied with spectroscopic methods.

Though pulse proteolysis can accurately determine C_m values, the method does not report accurate m -values, which is the dependence of $\Delta G_{\text{unf}}^\circ$ on the concentration of denaturant and a necessary parameter to determine $\Delta G_{\text{unf}}^\circ$. Even with sophisticated biophysical measurements, m -values are known to be difficult to determine accurately. The intrinsic errors from quantification from SDS-PAGE gels and the limited number of data points make the determination of m -values by pulse proteolysis impractical. For this reason, to determine $\Delta G_{\text{unf}}^\circ$ for soluble proteins by pulse proteolysis, we estimated the m -values from the size of the protein, based on the known statistical relationship.²⁸ Unfortunately, there is no empirical way to estimate m -values for membrane proteins. Without m -values, C_m values (or ΔC_m) cannot be converted into $\Delta G_{\text{unf}}^\circ$ (or $\Delta \Delta G_{\text{unf}}^\circ$). Still, C_m values are valuable in comparing the effect of mutation on the stability of proteins, as melting temperatures (T_m) are frequently used. Another potential application of C_m determination by pulse proteolysis is the monitoring of ligand binding to a membrane protein. A significant number of membrane proteins are hormone receptors and drug targets. Ligand binding to the native conformation will stabilize proteins, which results in an increase in C_m value. Therefore, C_m determination by pulse proteolysis in the presence of various ligands may be a convenient way to compare the affinity of ligands to a membrane protein in a quantitative manner. The application of pulse proteolysis as a tool to monitor ligand binding to membrane proteins is currently being developed in our laboratory.

Proteins frequently lose their activities due to the destabilizing effect of mutations far from their active sites. This is also common in mutations in integral membrane proteins that are known to be linked to human diseases.²⁹ The root of these dysfunctions stems from compromised stability rather than the disruption of the functional sites in the folded

protein. Experimental means to assess the destabilizing effect of the mutations are essential to decipher the molecular basis of these diseases. Pulse proteolysis coupled with Western blotting can be an innovative approach to evaluate the effects of pathogenic mutations on the stability of endogenous or transiently expressed membrane proteins in cell lysates without purification.

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