Volume 240, number 1,2, 211–215 FEB 06525 November 1988

Hydrogen exchange from the transbilayer hydrophobic peptide of glycophorin reconstituted in lipid bilayers

Malkit Sami and Christopher Dempsey

Biochemistry Department, Oxford University, South Parks Road, Oxford OX1 3QU, England

Received 26 September 1988

The hydrophobic transbilayer peptide of erythrocyte glycophorin has been purified following exchange of tritium into the backbone amides, and reconstituted in egg phosphatidylcholine micelles. Analysis of tritium exchange from the backbone amides of the membrane-reconstituted peptide shows that about two of the amides are virtually non-exchangeable, about 10 are slowed by factors of 10^7 relative to free amides in unstructured water soluble peptides and the remainder of the amides (about 20) have slowing factors of less than 1000. These classes of amides are proposed to reflect the stability of the peptide with respect to hydrogen bond breaking fluctuations and the accessibility of the amides to exchange catalysts in different regions of the bilayer.

Glycophorin; Transbilayer peptide; Amide exchange; Membrane protein; Reconstitution

1. INTRODUCTION

Analysis of single amide hydrogen exchange from peptide amides of soluble proteins by NMR gives high resolution information about intramolecular hydrogen-bonded structure, local conformational stability and backbone fluctuations involving hydrogen bond cleavage [1]. For membrane proteins the degree of detail obtained with water soluble proteins may be achieved for proteins solubilized in organic solvents [2] or within detergent micelles [3]. The analysis of amide exchange from membrane proteins in situ is limited to low-resolution methods including the measurement of bulk exchange of hydrogen for tritium or infrared spectroscopic analysis of hydrogen-deuterium exchange [4–8]. Studies of amide exchange from the retinal-containing proteins rhodopsin and bacteriorhodopsin have shown that for rhodopsin (but not bacteriorhodopsin) a significant number of side chain amides are protected from exchange presumably due to intramolecular hydrogen bond formation [5,7] and that the number of slowly exchanging amides in bacteriorhodopsin (71%) corresponds to the number of amides expected to be hydrogen bonded within the 7 putative transmembrane helices of the protein [8].

A question of interest is the mechanism of exchange of amides within a membrane protein. It is generally assumed that the minimal requirements for exchange of an intramolecularly hydrogen bonded amide are hydrogen bond breaking backbone fluctuations and exposure of the non-hydrogen-bonded amide to exchange catalyst (H^+ or OH^-) either by diffusion of the catalyst from bulk solution to the amide or by exposure of the amide to the bulk solution [1]. Each of these requirements is expected to be difficult for membrane proteins because the non-polar interior of the membrane should greatly suppress both the conformational fluctuations involving hydrogen bond cleavage and exposure of the polar amide NH

Correspondence address: C.E. Dempsey, Biochemistry Department, Oxford University, Oxford OX1 3QU, England

Abbreviations: bR, bacteriorhodopsin; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; PC, phosphatidylcholine; T(is), (insoluble) tryptic transbilayer peptide of glycophorin; TFA, trifluoroacetic acid; TFE, trifluoroethanol; TLC, thin layer chromatography

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50

and carbonyl groups and the diffusion of polar exchange catalyst into the membrane interior. Exchange from large membrane proteins might be enhanced by polar 'channels' formed within the structure of the protein that would allow hydrogen bond cleavage and diffusion of exchange catalyst. Exchange from a single membrane-spanning helical peptide might be expected to be greatly suppressed.

We report here an analysis of tritium exchange from the transbilayer hydrophobic peptide of erythrocyte glycophorin (T(is)) reconstituted in egg phosphatidylcholine bilayers. The peptide is proposed to span the membrane in a helical conformation [9,10]. Our aims are to determine whether amide exchange can occur from a transbilayer helical peptide within a membrane and whether information about the conformation, orientation and dynamics of the peptide in the membrane may be obtained from amide exchange measurements.

2. MATERIALS AND METHODS

Glycophorin was purified [11] from erythrocyte ghosts prepared [12] from recently outdated blood of no particular blood group preference and delipidated by extracting a 0.5 $mg \cdot ml^{-1}$ solution in 10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA with an equal volume of 50% phenol [13]. The glycophorin contained more than 90% glycophorin A with small amounts of glycophorin B when analyzed on 'Stains-all' gels [14], and after delipidation had less than 3 mol phospholipid per glycophorin monomer when analyzed for inorganic phosphate after acid hydrolysis [15].

The hydrophobic transbilayer peptide of glycophorin, T(is) [9], was prepared by treating glycophorin (20 $mg \cdot ml^{-1}$ in 50 mM Tris-HCl, pH 9.5) with TPCK-treated trypsin (5%, w/w) for 2 h at 37°C followed by acidification to pH 3 upon which the hydrophobic peptide precipitates [9]. The precipitate was recovered by centrifugation, washed twice in cold 100 mM sodium acetate, pH 4, freeze dried and stored at −20°C. For preparation of tritiated peptide the trypsinization was carried out on a sample of glycophorin in pH 9.5 buffer containing 25 $mCi \cdot ml^{-1}$ $[^3H]_2O$ after preincubating the protein for 24 h at 37°C in the same tritiated buffer.

The T(is) peptide was homogeneous on analysis by gel electrophoresis on urea gels [16] staining red in Stains-all (indicating the absence of carbohydrate) and blue in Coomassie with a single tailing band in the 2–4 kDa region. The peptide was about 80% pure by analysis on reverse-phase HPLC. Analysis of the major fraction by amino acid analysis gave the expected composition with the major inhomogeneity resulting from small amounts of N-terminally extended peptide (residues 62–101 of glycophorin rather than 62–96) as determined by the greater number of Lys and Arg residues in some fractions.

The T(is) peptide was reconstituted by mixing a solution of the peptide (1 mg) in 4 ml of 0.5% TFA in TFE, with 100 mg of egg PC in 5 ml of ethanol and drying aliquots of the mixture under reduced pressure with a diffusion pump. Tritium efflux from the reconstituted peptide was initiated by suspending the dried peptide-lipid complex in 5 ml of reconstitution buffer (200 mM constant ionic strength buffers of Miller and Golder [17]) of varying pH (see fig.2) at 0°C. The reconstituted lipid-peptide complexes were incubated at 25°C and aliquots were removed over a time course and immediately frozen in solid CO_2. The frozen complexes were freeze dried to remove tritium exchanged from the peptide into bulk water, and tritium remaining in the peptide was determined by scintillation counting after resuspension of the complexes in 0.1 ml of water. Reconstituted samples of the T(is) peptide in egg PC were checked by TLC for the presence of hydrolytic products (fatty acid and lysoPC); no lipid hydrolysis was detected after 4 weeks in the samples incubated at pH 5–9, but about 10% of the lipid was converted to lysoPC and fatty acid after 4 weeks at pH 3 and at pH 11.

3. RESULTS

Samples of T(is) peptide prepared by trypsinization of glycophorin in deuterated or protonated buffer were examined by high resolution NMR spectroscopy in TFE-d_3 (0.5% TFA-d_3) to determine the extent of isotope exchange into the back-

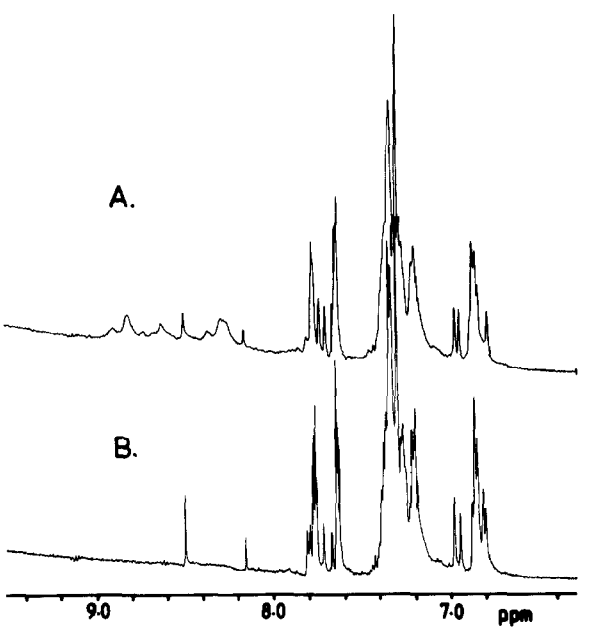

Fig.1. Low-field region of the 500 MHz proton NMR spectrum of the hydrophobic transbilayer peptide of glycophorin in perdeuterated trifluoroethanol. The peptide was prepared from glycophorin by trypsinization in protonated buffer (A), or deuterated buffer (B).

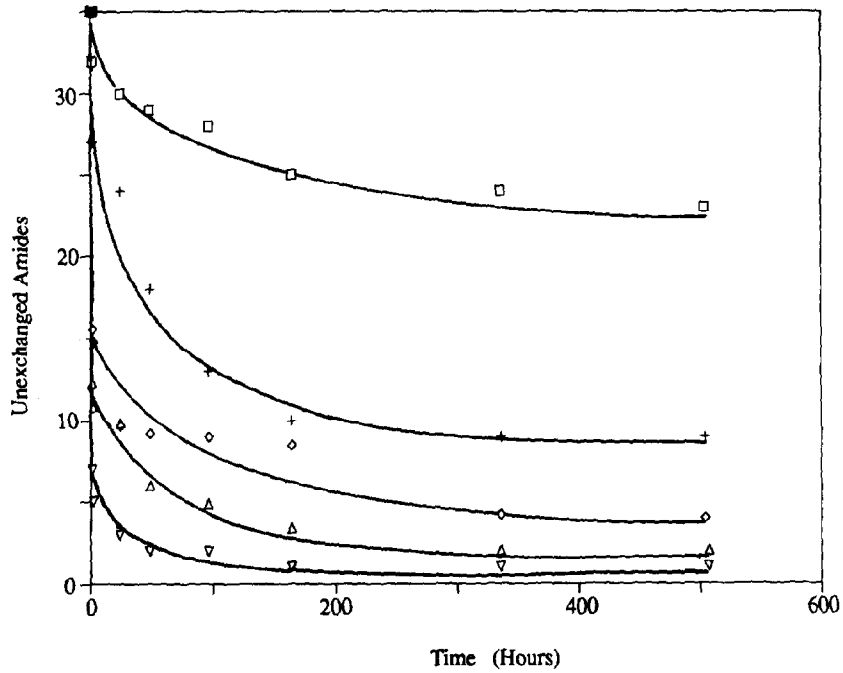

Fig.2. The pH-dependence of hydrogen exchange from the transbilayer hydrophobic peptide of glycophorin reconstituted in egg phosphatidylcholine bilayers. The curve shows the unexchanged amide equivalents remaining in the peptide at increasing times at pH 3 (□), 5 (+), 7 (◇), 9 (△), and 11 (▽). Lines are drawn to guide the eye.

bone amides of the peptide. The spectrum of the peptide prepared in deuterated buffer (fig.1B) shows the complete loss of signal intensity in the amide region of the NMR spectrum characteristic of α-helical amides (8–9.2 ppm) compared with preparations made by trypsinization of glycophorin in protonated buffer (fig.1A) indicating complete in-exchange of isotope into the backbone amides under identical conditions as used for amide tritiation.

The reconstituted lipid-peptide complexes (100:1 lipid/peptide, w/w) ran as single bands on sucrose density gradients with no evidence for aggregated (non-reconstituted) peptide that occurred at higher peptide/lipid ratios (> 10% by weight of peptide). The observed tritium out-exchange is from reconstituted peptide rather than from non-reconstituted aggregated peptide. The T(is) peptide is expected to be completely dissociated into monomers at this protein/lipid ratio [10], although we cannot discount the possibility that the predominant form of the peptide is a dimer [18].

pH-dependent tritium exchange from T(is) reconstituted in egg PC bilayers into buffered solutions of 200 mM ionic strength at 25°C is shown in fig.2. The out-exchange is generally increased with

Table 1

Estimates of the numbers of fast and slowly exchanging amides from T(is) peptide reconstituted in egg PC bilayers

pH	Fast ($t_{1/2}$ < 30 min)	Slow ($t_{1/2}$ > 100 h)	Amides unexchanged after time indicated at pH 7		
			M_{13}[a] (10 h)	bR[b] (10 h)	Melittin (5 min)
3	3– 5	23–25			
5	7– 9	10–12			
7	18–20	8–10	40%	71%	0
9	22–25	4– 5			
11	26–28	1– 2			

[a] NMR data for M_{13} coat protein in perdeuterated SDS micelles; from [3]

[b] Infrared data for bacteriorhodopsin in purple membranes; from [8]

increasing pH as expected from catalysis by OH^-. Each exchange sample was prepared by division of a single preparation of cosolubilized lipid and tritiated peptide. With the knowledge that under the isotopic labelling conditions complete in-exchange of tritium into the amides of T(is) was achieved (fig.1), exchange curves can be normalized with the zero-time tritium count being equivalent to about 35 amides (fig.2). The residual tritium count at any time, t, is readily convertible to the number of unexchanged amide 'equivalents' remaining in the peptide. Although attempts to fit the exchange data to double exponential decays were unsuccessful, there is some justification for dividing the exchanging amides into kinetic classes (table 1); i.e. those that exchange very quickly at a given pH (with $t_{1/2}$ less than 30 min, the first sampling point) and those that exchange very slowly (with $t_{1/2}$ greater than 100 h).

4. DISCUSSION

Amide exchange experiments performed by measuring tritium exchange into membrane proteins in situ are complicated by the uncertainty that all exchangeable sites are labelled at the end of the experiment [6,7]. In the present case we can be confident that the initial radioactivity at $t = 0$ corresponds to about 35 labelled sites containing equivalent counts and can thus accurately assess the count remaining in the peptide at any time during exchange-out in terms of the number of unexchanged amides. The latter number corresponds to amide equivalents because each exchange curve is a composite of a number of first order decays.

The exchange data demonstrate that while most of the amides of a transmembrane helical peptide can exchange with bulk solvent, exchange may be heavily suppressed and the protection of amides in different regions of the transmembrane peptide may vary considerably. It is expected that amides at the N- and C-terminal ends of a transbilayer helical peptide would exchange more quickly due to (i) helix tailing effects that promote fluctuations at helix termini [19] and (ii) the polar nature of the head group region of membranes allowing diffusion of exchange catalyst and solvation of polar amide and carbonyl groups exposed during hydrogen bond cleavage. In the reconstituted T(is) around 20 amides that exchange with slowing factors of less than 1000 relative to PDLA at pH 7 are probably located in the terminal regions of the helix at or near the membrane surface where the major limit to exchange may be the frequency of helix-opening fluctuations rather than access of the exchange catalyst. Around 10 amides are very difficult to exchange from the reconstituted peptide (with slowing factors of 10^7-fold relative to PDLA) and these probably correspond to amides located within the central 1.5 nm of the membrane. About two of these amides are virtually non-exchangeable and this very stable class (slowing factors of $>10^{11}$-fold) may be hydrogen bonded within the helix at the centre of the bilayer. These data are consistent with the proposed [10,18,20] transbilayer helical conformation of the T(is) peptide. By comparison, all the backbone amides exchange from the membrane-bound hemolytic peptide melittin (26 amino acids) within 5 min at pH 7 (not shown). Melittin probably lies along the membrane surface [21], and the large differences in exchange protection of amides within T(is) relative to melittin (factors of up to 10^7-fold) are almost certainly due to the different orientations of these peptides within membranes.

The exchange data for the transbilayer helical peptide of glycophorin can be compared with other exchange data for membrane proteins (table 1). Amide exchange from the hydrophobic core of the helical M_{13} coat protein in SDS micelles has been studied by high resolution NMR spectroscopy and a stable core of around 20 amides having exchange rates suppressed by 10^4 relative to PDLA were found [3]. The much larger slowing factors found in the present study are probably an indication of the restraints on both conformational dynamics and exchange catalyst accessibility that occur for a membrane-incorporated peptide compared with a detergent solubilized one. In the case of bacteriorhodopsin a much greater proportion of amides (70%) are slow at pH 7, a number corresponding to all the amides in the putative transmembrane helices. As with other soluble proteins, solvent accessible helical segments may exhibit slow amide exchange due to the limitations on hydrogen-bond breaking conformational fluctuations imposed by the overall structural stability of the protein [1]. The less extensive core of stable amides (around 30%) in the transmembrane helical peptide of

glycophorin is expected from the lack of restraints on conformational dynamics at the helix termini.

Acknowledgements: We are grateful to Dr A. Watts for his interest and the use of laboratory facilities, to the Oxford Enzyme Group for use of the WH-500 NMR spectrometer, and to the Nuffield Foundation and the Research and Equipment Committee of Oxford University for grants to C.D. in support of this research.

REFERENCES

[1] Englander, S.W. and Kallenbach, N.R. (1984) Q. Rev. Biophys. 16, 521–655.
[2] Dempsey, C.E. (1988) Biochemistry 27, 6893–6901.
[3] O'Neil, J.D.J. and Sykes, B.D. (1988) Biochemistry 27, 2753–2762.
[4] Downer, N.W. and Englander, S.W. (1977) J. Biol. Chem. 252, 8092–8100.
[5] Osborne, H.B. (1976) FEBS Lett. 67, 23–27.
[6] Osborne, H.B. and Nabedryk-Viala, E. (1977) FEBS Lett. 84, 217–220.
[7] Englander, J.J., Downer, N.W. and Englander, S.W. (1982) J. Biol. Chem. 257, 7982–7986.
[8] Downer, N.W., Bruchman, T.J. and Hazzard, J.H. (1986) J. Biol. Chem. 261, 3640–3647.
[9] Segrest, J.P., Kahane, I., Jackson, R.L. and Marchesi, V.T. (1973) Arch. Biochem. Biophys. 155, 167–183.
[10] Segrest, J.P. (1977) in: Mammalian Cell Membranes (Jamieson, G.A. and Robinson, D.M. eds) vol. 3, pp. 8–26, Butterworth & Co., London.
[11] Hamaguchi, H. and Cleve, H. (1972) Biochim. Biophys. Acta 278, 271–280.
[12] Dodge, J.T., Mitchell, C. and Hanahan, D.J. (1963) Arch. Biochem. Biophys. 100, 119–130.
[13] Van Zoelen, E.J.J., Zwaal, R.F.A., Reuvers, F.A.M., Demel, R.A. and Van Deenen, L.L.M. (1977) Biochim. Biophys. Acta 464, 482–492.
[14] King, L.E. and Morrison, M. (1976) Anal. Biochem. 71, 223–230.
[15] Rouser, G., Fleischer, S. and Yamamoto, A. (1970) Lipids 5, 494–496.
[16] Swank, R.T. and Munkries, K.D. (1971) Anal. Biochem. 39, 462–477.
[17] Miller, G.L. and Golder, R.H. (1950) Arch. Biochem. 29, 420–423.
[18] Ross, A.H., Radhakrishnan, R., Robson, R.J. and Khorana, H.G. (1982) J. Biol. Chem. 257, 4152–4161.
[19] Nakanishi, M., Masamichi, T., Ikegami, A. and Kanehisa, M. (1972) J. Mol. Biol. 64, 363–378.
[20] Welsh, E.J., Thom, D., Morris, E.R. and Rees, D.A. (1985) Biopolymers 24, 2301–2332.
[21] Terwilliger, T.C., Weissman, L. and Eisenberg, D. (1982) Biophys. J. 37, 353–361.