J. Biochem. 124, 417-420 (1998)

Treatment with Crystalline Ultra-Pure Urea Reduces the Aggregation of Integral Membrane Proteins without Inhibiting N-Terminal Sequencing[1]

Stéphanie Soulié,* Luc Denoroy,[†,2] Jean-Pierre Le Caer,[‡] Naotaka Hamasaki,[§] Jonathan D. Groves,[‖] and Marc le Maire*[,3]

*Section de Biophysique des Protéines et des Membranes, Département de Biologie Cellulaire et Moléculaire, CEA et CNRS URA 2096, CEA de Saclay, 91191 Gif sur Yvette Cedex, France; [†]Service Central d'Analyse, CNRS B.P 22, 69390 Vernaison, France; [‡]Laboratoire de Neurobiologie, Ecole Supérieure de Physique et de Chimie Industrielles de la Ville de Paris, CNRS URA 2054, 10 rue Vauquelin, F-75231 Paris Cedex 05, France; [§]Department of Clinical Chemistry and Laboratory Medicine, Kyushu University Faculty of Medicine, Fukuoka 812-82, Japan; and [‖]Department of Biochemistry, School of Medical Sciences, University of Bristol, Bristol BS8 1TD, United Kingdom

Received for publication, March 20, 1998

We have demonstrated that N-terminal sequencing can be performed successfully despite boiling protein samples in the presence of urea under precise conditions, before loading them onto SDS-PAGE and transfer to polyvinylidene difluoride membrane. Using myoglobin as a test protein, we found that its ability to undergo N-terminal sequencing was not affected by the presence of urea provided "ultra-pure" urea was used. Consistent with this result, we verified that urea did not carbamylate myoglobin since its molecular mass was measured by mass spectrometry after electroelution of the protein band from the gel. These observations are useful for the study of integral membrane proteins, in particular to study their topology from proteolysis experiments, since heating in the presence of urea before SDS-PAGE reduces membrane protein aggregation [Soulié, S., Møller, J.V., Falson, P., and le Maire, M. (1996) *Anal. Biochem.* 236, 363-364]. We show that the sequencing yield of a hydrophobic peptide from reticulum Ca^{2+}-ATPase was more than doubled in the presence of urea in accord with the quantification of the Coomassie Blue staining of the gel and of the amount present on the polyvinylidene difluoride membrane. For three peptides of the gastric H^+K^+-ATPase, the sequencing yield after urea treatment increased almost threefold.

Key words: carbamylation, membrane protein, N-terminal sequencing, topology, urea.

Proteolytic digestion of integral membrane proteins *in situ* is commonly used for investigation of their topology or tight folding. For analysis of these samples by SDS-PAGE, it is essential that the protease is completely inactivated to avoid spurious results. Although heating in Laemmli gel sample buffer containing SDS and 2-mercaptoethanol at 100°C is effective, it has been recognized for many years that such treatment tends to promote the aggregation of a wide variety of membrane proteins and their peptide fragments (*1*). Alternative means of avoiding membrane protein aggregation, such as denaturation for more extended periods at lower temperatures, or the use of trifluoroacetic acid, are effective in some situations (*1*). Using p19, a four transmembrane span fragment derived from proteinase K digestion of the sarcoplasmic reticulum Ca^{2+}-ATPase (*2*), we have shown in a previous report (*3*) that addition of crystalline urea to the sample in SDS buffer (to give a final concentration of approx. 8 M urea) will permit heating without promoting aggregation. Since low molecular weight, hydrophobic products of proteolysis are particularly prone to aggregate, urea treatment is a simple and effective procedure that discourages selective losses of peptides.

In this paper we show that myoglobin, as a test protein, and hydrophobic proteolytic fragments that have been treated with ultra-pure urea are suitable for N-terminal sequencing by Edman degradation. Protein sequencing is an important technique for the identification of proteolytic fragments and one which many people have considered to be incompatible with the exposure of samples to urea. Urea breaks down slowly in aqueous solution to form cyanate, which may carbamylate the N-terminal amino acid residue of the peptide and hence block sequencing (see Refs. *4-6*). We show that the N-terminal sequencing of several of the membrane protein fragments is not affected by the addition of crystalline ultra-pure urea in the protease inhibition step. Furthermore, we show that the reduced levels of aggregation obtained in the presence of urea contribute to

[1] This work was supported by the CEA, CNRS, the Wellcome Trust and a grant from the Association Francaise contre les Myopathies (to M. le M.).

[2] Present address: Laboratoire de Neuropharmacologie et Neurochimie, Faculté de Pharmacie, Université Claude Bernard-Lyon I, 8 avenue Rockfeller, 69373 Lyon Cedex 08, France.

[3] To whom correspondence should be addressed. Tel: +33-169086243, Fax: +33-169088139, E-mail: lemairem@dsvidf.cea.fr

Abbreviations: PVDF, polyvinylidene difluoride; SR, sarcoplasmic reticulum.

higher signals in the first and subsequent rounds of protein sequencing.

MATERIALS AND METHODS

Chemicals—Ultra-pure grade urea (Aristar) was purchased from BDH; analytical grade urea (pro analysi) from Merck; SDS from Biorad, PVDF membrane filter (Immobilon-P) from Millipore; acrylamide/bisacrylamide (ultrapure Protogel) from National Diagnostics; horse skeletal muscle myoglobin from Sigma (ref. M0630), proteinase K from Boehringer Mannheim.

Membrane Proteins and Peptides Preparation—Rabbit SR Ca^{2+}-ATPase from skeletal muscle was prepared and digested by proteinase K as described in Ref. *2*. The purification of pig gastric H^+K^+-ATPase was described in Ref. *7*, the membrane suspension in 125 mM bis-Tris, pH 6.5, 0.62 mM $CaCl_2$ was treated with proteinase K as described in the figure legend.

Methods—SDS/PAGE, Western blotting, and immunodetection were as in Ref. *2*. The treatment of the samples in urea before loading onto SDS/PAGE was as follows (*3*): 15 μl of a mixture containing 6.7% (w/v) SDS and 4.6 M 2-mercaptoethanol (approximate pH of the mixture: 3) was added to 40 mg of crystalline urea; vortexing dissolved only part of the urea; the protein sample (40 μl, 1-20 μg) was added and the vortexing dissolved the rest of the urea; the sample was then heated at 100°C for 70 s; the entire process until loading the gel did not take longer than five minutes. SDS-PAGE gels were either stained with Coomassie Blue or proteins separated on SDS-PAGE gels were transferred onto PVDF membrane (Immobilon P from Millipore) by electroblotting for 90 min at 500 mA (*8*). Membranes were immunostained with polyclonal anti-peptide antibody directed against aa residues 877-888 [which detects p19 sequences (*2, 9*)], and detected by enhanced chemiluminescence (Amersham, UK). Scanning densitometry was performed in the linear exposure range using different quantities of protein on gels and various exposure periods on a GS-700 Imaging Densitometer (BioRad), and the digitized images were treated with Molecular Analyst software (BioRad).

Edman degradation was performed using an Applied Biosystems 470-A gas-phase sequencer with on-line PTH analysis. In each case the first six amino acids were sequenced. The amount of material that gave a detectable sequence in the first cycle is presented. The results of each of the five subsequent cycles of sequencing were similar to those of the first cycle. The overall recovery (*i.e.*, considering both the recovery after transfer on the blot and the initial yield from the sequencing instrument) from the first cycle was within the normal expected range (*8*). Electrospray ionization mass spectrometry and electroelution from gels were as in Ref. *10*, with the modifications described in Ref. *2*.

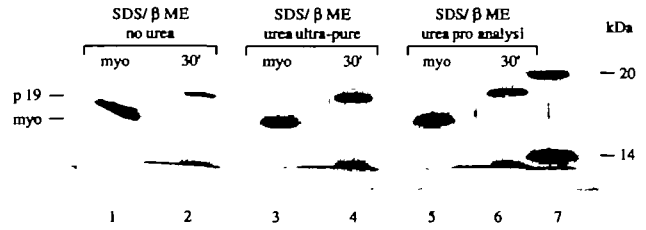

Fig. 1. **Effect of urea treatment on the aggregation of p19, a hydrophobic fragment derived from proteinase K digestion of the Ca^{2+}-ATPase.** Total protein (14 μg) from sarcoplasmic reticulum (SR) vesicles in 100 mM bis-Tris, pH 6.5, 0.1 mM Ca^{2+} was digested by proteinase K for 30 min (lanes 2, 4, and 6) at 20°C. Samples (40 μl) were combined with 15 μl of 6.7% (w/v) SDS, 4.6 M 2-mercaptoethanol and heated at 100°C for 70 s as described in Ref. *3* either in the absence of urea (lanes 1 and 2) or the presence of 8 M "ultra-pure" grade urea (BDH Aristar; lanes 3 and 4) or 8 M "analytical" grade urea (Merck pro analysi; lanes 5 and 6). SDS-PAGE was performed on 12.5% gels followed by staining with Coomassie Blue. 14 μg of SR vesicles contains about 11 μg of Ca^{2+}-ATPase, which yields about 1 μg of p19 after proteinase K digestion (*2*). The p19 fragment is shown in lanes 2, 4, and 6. Control lanes were loaded with 2 μg of horse myoglobin (lanes 1, 3, and 5).

RESULTS AND DISCUSSION

We have examined the suitability of urea-treated membrane protein fragments for N-terminal sequencing by Edman degradation. p19 is a hydrophobic integral membrane fragment of the Ca^{2+}-ATPase containing the C-terminal four putative transmembrane spans (aa residues D818-G994) that is obtained by digestion of the intact protein with proteinase K (*2*). Figure 1 shows the effect of urea on SDS-PAGE of p19, and of myoglobin as a globular protein control, focusing on the low M_r region of the gel. Samples (myoglobin or Ca^{2+}-ATPase after proteolysis for 30 min) were either heated (lanes 1 and 2), or heated in the presence of one of two grades of urea: either a high quality "ultra-pure" grade (lanes 3 and 4) or a lower quality "pro analysi" (analytical) grade (lanes 5 and 6), as detailed in the figure legend. Table Ia shows the results of scanning densitometry of several Coomassie Blue-stained gels: the p19 band in the urea-treated samples (Fig. 1, lanes 4 and 6) contained about twice the amount of this polypeptide compared with the sample that was heated without urea (lane 2). In contrast, myoglobin was unaffected by the presence or absence of urea (Table Ia and Fig. 1, lanes 1, 3, and 5).

SDS-PAGE gels containing the p19 prepared by proteinase K were electroblotted onto PVDF membranes and immunostained with polyclonal antibody raised against synthetic peptide to p19 [aa residues 877-888 (*2, 9*)]. Scanning densitometry of quantitative anti-p19 blots showed that the ultra-pure urea treated sample contained about twice the amount of unaggregated p19 of the samples that were heated without urea (Table Ib), as in the Coomassie Blue-stained gels (Table Ia).

Both p19 and myoglobin were excised from the PVDF

TABLE I. **Effect of urea on the quantity of myoglobin and p19 detected on SDS-PAGE gels by Coomassie Blue staining ([a]), and of p19 on PVDF membrane by Western immunoblotting ([b]).** Values (arbitrary units) determined by scanning densitometry for urea-treated and untreated samples are mean ± standard deviation. Key: n.d., not determined. Note that peptide p19 appears to take up the Coomassie Blue stain less strongly than myoglobin.

	No urea	Ultra-pure urea	Analytical grade urea
[a]Myoglobin (2 μg)	32.4±1.9	33.3±1.3	30.2±2.3
[a]p19 (*ca.* 1 μg)	3.7±0.3	7.3±0.2	6.0±0.6
[b]p19	1.0	2.4±0.5	n.d.

membrane and six cycles of Edman degradation were performed in each case. The results of N-terminal sequencing are shown in Table II. The ability of myoglobin to undergo N-terminal sequencing was not affected by the presence or absence of "ultra-pure" urea, although the strength of the signal from myoglobin samples treated with "analytical" grade urea was lower than in the other two treatments. In the case of p19, there was a marked increase in the strength of signal from the sequencing using both grades of urea compared with untreated samples. This probably derives from the greater quantity of unaggregated p19 transferred to the blots in the presence of urea (Table Ib). As observed with myoglobin, the sequencing signal was slightly stronger in the case of samples treated with "ultra-pure" rather than "analytical" grade urea, which indicates that the lower purity reagent exherts a slightly inhibitory effect on the sequencing of both proteins. To confirm that proteins treated with crystalline ultra pure urea were not carbamylated, the myoglobin band was electroeluted from the gel and analysed by electrospray ionization mass spectrometry (*10*). Figure 2 shows that the spectra are very similar with or without urea treatment and that the molecular mass obtained corresponds to that of native protein in each case.

We verified our sequencing results with p19 using three proteinase K fragments from the pig H^+K^+-ATPase. These fragments were derived from a different membrane protein and are of a different size (about 91, 54, and 28 kDa) to p19. Samples were heated in the presence or absence of ultra-pure urea prior to SDS-PAGE (Fig. 3). In the absence of urea, both digested and undigested α subunit of the H^+K^+-ATPase was aggregated by heating (see top of the gel, lanes 6-10), whereas urea-containing samples did not aggregate appreciably (lanes 1-5). The aggregation induced by heating in the absence of urea is even more obvious than in the case of the Ca^{2+}-ATPase (compare Fig. 1 of Ref. *3* and Fig. 3). After SDS-PAGE carried out as shown in Fig. 3, the proteins were electroblotted onto PVDF, and three peptides that appeared in fairly large amount as a result of H^+K^+-ATPase proteinase K treatment were subjected to Edman degradation (p91, p54, p28, see open arrows in Fig. 3). The initial yield of these three polypeptides was respectively 1.6, 4.5, and 2.2 pmol in the presence of ultra-pure urea and <0.5, 1.6, and 0.8 pmol in the absence of urea. Hence, the yield from sequencing increased at least twofold in the presence of ultra-pure urea, similarly to our result with p19 (Table II). Clearly, in our hands, heating in the presence of urea is required to inactivate the proteases and to increase the sequencing yield to a level well above background, and is therefore mandatory for obtaining reliable data in probing H^+K^+-ATPase topology by proteolysis.

With another membrane protein, the anion exchanger (band 3), urea treatment has been shown to reduce aggregation [yeast-expressed band 3 (*11*, data not shown) or a proteolytic fragment (*12*)], and not to prevent the Edman degradation: in a previous report (*13*), Kawano *et al.* treated a number of proteolytic fragments of the membrane domain of band 3 purified from human red cells with ultra-pure urea and this did not inhibit N-terminal se-

TABLE II. **Effect of urea on the N-terminal sequencing of myoglobin and p19 from PVDF membrane.** Protein bands corresponding to myoglobin and p19 were excised from PVDF membrane and analysed by Edman degradation. For myoglobin, the results represent the mean of at least two experiments; the standard deviation of the first six amino acid residues sequenced was about 7% of the mean value in three experiments. For p19, the material from at least two bands was combined and sequenced in a single experiment. For myoglobin and p19, each sample loaded on the gel contained 2 μg (120 pmol) or 1 μg (50 pmol) protein, respectively.

	Sequencing peptide (pmol)		
	No urea	Ultra-pure urea	Analytical grade urea
Myoglobin	19.0±1.8	19.1	13.9±1.4
p19	2.0	5.5	4.3

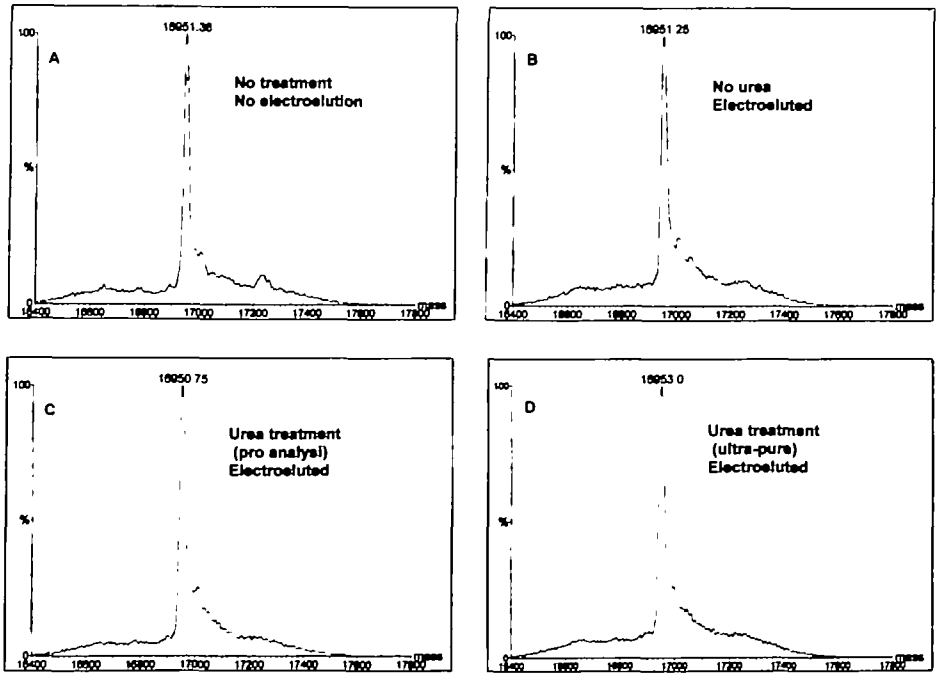

Fig. 2. **Deconvoluted ESI mass spectrum of myoglobin with or without urea treatment.** Panel A: 1 μg of myoglobin was injected in 10 μl of carrier solvent. Panels B, C, D: 0.2-1 μg of protein was electroeluted from SDS-PAGE according to Ref. *10* and injected in 10 μl of carrier solvent. The protein loaded on SDS-PAGE was either untreated (panel B) or treated with Merck pro analysi urea (panel C) or BDH ultra-pure urea (panel D) according to the legend to Fig. 1. The predicted M_r of myoglobin is 16,951.5 while the predicted M_r of myoglobin carbamylated on the N-terminal amino acid is 16,994.5.

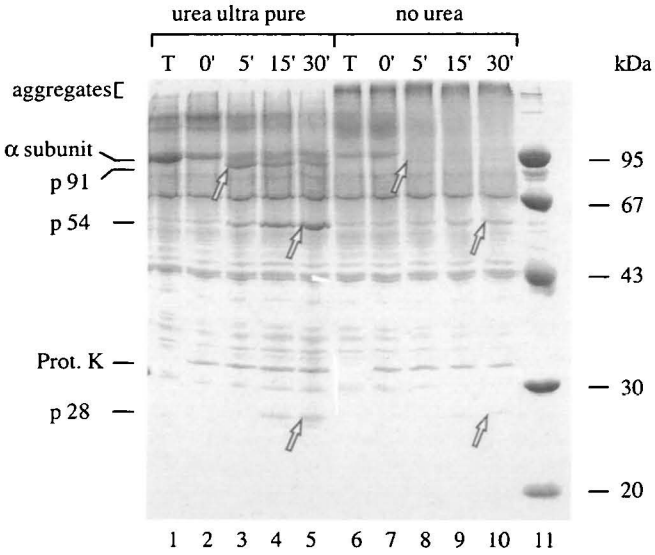

Fig. 3. **Effect of urea treatment on the aggregation of three peptide fragments of the H^+K^+-ATPase.** SDS-PAGE (11.4% gels) was performed using tubulovesicles (T) isolated from hog gastric fundus (7). Membrane suspension (1 mg protein/ml in 125 mM bis-Tris, pH 6.5, 0.62 mM $CaCl_2$) was treated with proteinase K (0.03 mg/ml) for 0 min (lanes 2 and 7), 5 min (lanes 3 and 8), 15 min (lanes 4 and 9), or 30 min (lanes 5 and 10) at 20°C, or untreated (lanes 1 and 6). Proteolysis was stopped as previously described (2). Samples (20 μl) were combined with 7.5 μl of 6.7% (w/v) SDS, 4.6 M 2-mercaptoethanol and heated at 100°C for 70 s as described in Ref. 3 either in the absence of urea (lanes 1 to 5) or the presence of 20 mg of "Ultra-pure" grade urea (BDH Aristar; lanes 6 to 10). The gel was stained with Coomassie Blue. Following a similar SDS-PAGE, proteins were electroblotted onto Immobilon P (Millipore) and Edman degradation was performed on the three peptides (p91, p54, and p28) indicated by arrows.

quencing. Although not stated in the paper, the authors of this work considered the use of a freshly prepared solution of ultra-pure grade urea to be essential for their success in sequencing. Combining these results with our own, we conclude that the addition of crystalline "ultra pure" urea combined with heating for a precise and relatively short time (70 s) before loading onto SDS-PAGE gels is likely to be a generally effective method for the preparation of aggregation-prone membrane protein fragments for N-terminal sequencing. We suggest that this procedure reduces aggregation of some of the most susceptible fragments, offers improved quantitative reliability of proteolysis experiments, and, importantly, does not affect the ability of these fragments to undergo subsequent N-terminal sequencing.

The assistance of P. Falson, A. Gomez de Gracia is gratefully acknowledged. We are indebted to Drs. Vincent Raussens and Erik Goormaghtigh (Université Libre de Bruxelles, Belgique) for providing us with a preparation of hog gastric tubulovesicles.

REFERENCES

1. Hennessey, J.P. Jr. and Scarborough, G.E. (1989) An optimized procedure for sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of hydrophobic peptides from an integral membrane protein. *Anal. Biochem.* **176**, 284-289
2. Juul, B., Turc, H., Durand, M.L., Gomez de Gracia, A., Denoroy, L., Møller, J.V., Champeil, P., and le Maire, M. (1995) Do transmembrane segments in proteolyzed sarcoplasmic reticulum Ca^{2+}-ATPase retain their functional Ca^{2+}-binding properties after removal of cytoplasmic fragments by proteinase K? *J. Biol. Chem.* **270**, 20123-20134
3. Soulié, S., Møller, J.V., Falson, P., and le Maire, M. (1996) Urea reduces the aggregation of membrane proteins on SDS-PAGE. *Anal. Biochem.* **236**, 363-364
4. Stark, G.R., Stein, W.H., and Moore, S. (1960) Reactions of the cyanate present in urea with amino acids and proteins. *J. Biol. Chem.* **235**, 3177-3181
5. Hagel, P., Gerding, J.J.T., Fieggen, W., and Bloemendal, H. (1971) Cyanate formation in solutions of urea. I. Calculation of cyanate concentrations at different temperature and pH. *Biochim. Biophys. Acta* **243**, 366-373
6. Matsudaira, P. (1989) Introduction in *A Practical Guide to Protein and Peptide Purification for Microsequencing* (Matsudaira, P.T., ed.) pp. 1-13, Academic Press, San Diego
7. Raussens, V., Ruysschaert, J.-M., and Goormaghtigh, E. (1997) Fourier transform infrared spectroscopy study of the secondary structure of the gastric H^+,K^+-ATPase and its membrane-associated proteolytic peptides. *J. Biol. Chem.* **272**, 262-270
8. Legendre, N. and Matsudaira, P. (1989) Purification of proteins and peptides by SDS-PAGE in *A Practical Guide to Protein and Peptide Purification for Microsequencing* (Matsudaira, P.T., ed.) pp. 49-69, Academic Press, San Diego
9. Møller, J.V., Ning, G., Maunsbach, A.B., Fujimoto, K., Asai, K., Juul, B., Lee, Y.-J., Gomez de Gracia, A., Falson, P., and le Maire, M. (1997) Probing of the membrane topology of sarcoplasmic reticulum Ca^{2+}-ATPase with sequence specific antibodies: evidence for plasticity of the C-terminal domain. *J. Biol. Chem.* **272**, 29015-29032
10. le Maire, M., Deschamps, S., Møller, J.V., Le Caer, J.P., and Rossier, J. (1993) Electrospray ionization mass spectrometry on hydrophobic peptides electroeluted from SDS-PAGE. Application to the topology of the sarcoplasmic reticulum Ca^{2+} ATPase. *Anal. Biochem.* **214**, 50-57
11. Groves, J.D., Falson, P., le Maire, M., and Tanner, M.J.A. (1996) Functional cell surface expression of the anion transport domain of human red cell band 3 (AE1) in the yeast *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA* **93**, 12245-12250
12. Kawano, Y. and Hamasaki, N. (1986) Isolation of a 5,300-Dalton peptide containing a pyridoxal phosphate binding site from the 38,000-Dalton domain of Band 3 of human erythrocyte membranes. *J. Biochem.* **100**, 191-199
13. Kawano, Y., Okubo, K., Tokunaga, F., Mitaya, T., Iwanaga, S., and Hamasaki, N. (1988) Localization of the pyridoxal phosphate binding site at the COOH-terminal region of erythrocyte Band 3 protein. *J. Biol. Chem.* **263**, 8232-8238