BIOCHEMISTRY
including biophysical chemistry & molecular biology
Rapid Report

Biochemistry **2010,** *49,* 10589–10591 10589
DOI: 10.1021/bi101840j

Novel Hydrophobic Standards for Membrane Protein Molecular Weight Determinations via Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis†

Arianna Rath,‡ Vincent G. Nadeau,‡,§ Bradley E. Poulsen,‡,§ Derek P. Ng,‡,§ and Charles M. Deber*,‡,§

‡*Division of Molecular Structure and Function, Research Institute, The Hospital for Sick Children, Toronto, Ontario, Canada M5G 1X8, and* §*Department of Biochemistry, University of Toronto, Toronto, Ontario, Canada M5S 1A8*

Received November 18, 2010; Revised Manuscript Received November 24, 2010

ABSTRACT: Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) is a universally employed technique that separates proteins on the basis of molecular weight (MW). However, membrane proteins are known to size anomalously on SDS−PAGE calibrated with conventional standards, an issue that complicates interpretation of protein identity, purity, degradation, and/or stoichiometry. Here we describe the preparation of novel polyleucine hydrophobic standards for SDS−PAGE that reduce the average deviation of the apparent MW from the formula MW of natural membrane proteins to 7% versus 20% with commercially available standards. Our results suggest that gel calibration with hydrophobic standards may facilitate the interpretation of membrane protein SDS−PAGE experiments.

A wide variety of protein analysis procedures utilize sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) to separate polypeptides on the basis of molecular weight (MW). While SDS−PAGE is not intended for high-resolution MW determination, nearly all of its applications require a reasonable estimate of apparent MW. Such experiments include but are not limited to protein expression and/or purification trials, evaluation of protein degradation, immunoprecipitation experiments in which cross-reaction of an antibody and/or truncation or modification of a target protein is an issue, and/or identification of the stoichiometry of protein complexes. MWs are estimated via SDS−PAGE by comparison of the migration distance through the gel of an analyte protein(s) to a calibration curve derived from the MW values and gel migration distances of protein standards. The accuracy of these determinations requires that analytes and standards exhibit comparable gel separation behavior (*1*, *2*). Analyte proteins that sieve differently through gels than calibration reagents may display apparent MW values via SDS−PAGE that do not correspond to the actual MW, a phenomenon often termed "gel shifting".

Among the analytes known for decades to exhibit gel shifts on SDS−PAGE are helical integral membrane proteins. The high hydrophobicity and associated detergent binding levels of these molecules versus the overwhelming majority of calibration standards (typically mixtures of globular water-soluble proteins and/or polypeptides; for two exceptions, see refs *3* and *4*) can lead to SDS−PAGE readouts that deviate from the actual MW by up to ~50% (*5*), behavior that can complicate the interpretation of any laboratory procedure requiring an SDS−PAGE analysis. Given the popularity of SDS−PAGE as an experimental technique, its low capital costs versus other protein sizing approaches (*6*), its indispensability in co-immunoprecipitation experiments and proteomics (*7*), and the pharmaceutical importance of membrane proteins (*8*, *9*), an improvement in the reliability of membrane protein apparent MW estimates via SDS−PAGE should prove to be useful to the research community.

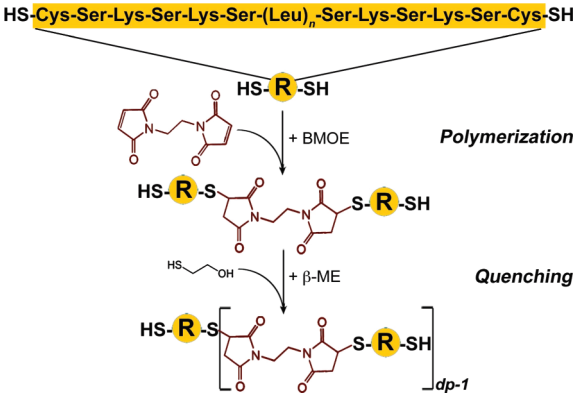

FIGURE 1: Polymerization scheme. The peptide is abbreviated as R, and *dp* is the number of peptide units in a given polymer. Cross-linking was performed at a peptide concentration of 200 μM in an aqueous solution of 80% (v/v) 2,2,2-trifluoroethanol (TFE) buffered at pH 7.5 with 20 mM Tris-HCl. Polymerization was terminated with β-mercaptoethanol (β-ME). Peptide synthesis and cross-linking reaction details are provided in the Supporting Information.

With this in mind, we designed and produced hydrophobic standards for SDS−PAGE shown herein to improve the reliability of apparent MW determinations of membrane proteins compared to state-of-the-art products. These "poly-Leu" standards consist of hydrophobic peptides specifically created to resemble the general layout of helical transmembrane (TM) proteins. Their component peptides have the base sequence H_2N-Cys-Ser-Lys-Ser-Lys-Ser-(Leu)$_n$-Ser-Lys-Ser-Lys-Ser-Cys-NH_2, where n is the number of Leu residues. The poly-Leu peptide core mimics the high hydrophobicity and abundance of Leu in natural helical TM sequences, while the basic and hydrophilic terminal residues resemble the residues common to TM segment ends and TM-linking regions (*10*).

Our production scheme utilizes the sulfhydryl moiety of Cys for peptide self-polymerization, achieved in a "one-pot" reaction by cross-linking following the scheme in Figure 1.

†This work was supported, in part, by grants to C.M.D. from the Canadian Institutes of Health Research (CIHR Grant FRN-5810) and the MaRS Innovation Proof of Principle Program. V.G.N. holds a Bourse de formation de doctorat from the Fonds de la Recherche en Santé du Québec. B.E.P. holds a Research Training Centre (RESTRACOMP) Award from The Hospital for Sick Children. D.P.N. holds a Frederick Banting and Charles Best Canada Graduate Scholarship from CIHR.

*To whom correspondence should be addressed: Division of Molecular Structure and Function, Research Institute, The Hospital for Sick Children, 555 University Ave., Toronto, Ontario, Canada M5G 1X8. Telephone: (416) 813-5924. Fax: (416) 813-5005. E-mail: deber@sickkids.ca.

Bis-maleimidoethane (BMOE) was selected as a conjugating linkage because of the sulfhydryl specificity of its reaction at neutral pH, the stability of the resulting thioether linkage to changes in pH and to reducing agents, the trans configuration of linkages, and the commonality of thioether conjugates in biomaterials (*11*).

A peptide with a sufficient number of Leu residues to span a typical bilayer as an α-helix (*10*) [$n = 20$ (Leu_{20})] was synthesized, purified, and self-polymerized (see the Supporting Information for details). After the reaction had been quenched, products were subjected to SDS−PAGE directly from the reaction mixture at a concentration of ∼200 ng/gel lane and visualized by silver staining (Figure 2, lane 1). The cross-linked poly-Leu_{20} peptide separated into a ladder of sharp bands with degrees of polymerization of 1−11 peptide units, translating into MWs of 3.5−41 kDa. The MW range achievable with the poly-Leu_{20} product could therefore be applied to SDS−PAGE of hydrophobic peptides and/or low-MW membrane proteins and was comparable to those of commercial "peptide" and "low-MW" products. Further optimization of reaction conditions is anticipated to yield poly-Leu_{20} species with MWs suitable for larger proteins.

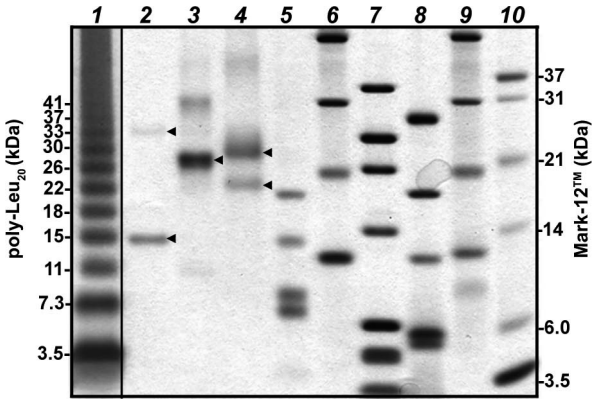

FIGURE 2: Representative SDS−PAGE. Lane 1: poly-Leu_{20} standards. Lane 2: EmrE. Lane 3: Hsmr. Lane 4: PLP (top band) and DM-20 (bottom band). Lane 5: Amersham Peptide SDS−PAGE Standards. Lane 6: Amersham Low Molecular Weight SDS−PAGE Standards. Lane 7: PageRuler Unstained Low Range Protein Ladder. Lane 8: Bio-Rad Polypeptide SDS−PAGE Standards. Lane 9: Bio-Rad Low-Range SDS−PAGE Standards. Lane 10: Mark-12 Unstained Standard. The migration position and MW in kilodaltons of poly-Leu_{20} bands (left) and Mark-12 standards (right) are indicated. Note that poly-Leu_{20} polypeptides migrate slower than commercial products at low MW values, like commercial products at ∼15 kDa, and more rapidly than commercial products at larger MWs. Arrows indicate the migration position(s) of membrane protein band(s) used to determine the apparent MW. SDS−PAGE was performed on 12% NuPAGE Bis-Tris gels purchased from Life Technologies. See the Supporting Information for experimental details.

To determine if the poly-Leu hydrophobic standards were more effective in estimating the apparent MWs of natural helical membrane proteins than reagents from various suppliers, several products (Amersham Peptide and LMW, Fermentas PageRuler Low Range, Bio-Rad Polypeptide and LMW, and Mark-12) were electrophoresed in parallel with poly-Leu_{20} and with four natural helical membrane proteins available in our laboratory (Figure 2). The proteins utilized [the *Escherichia coli* small multidrug resistance protein (EmrE), the *Halobacterium salinarum* small multidrug resistance protein (Hsmr), the bovine myelin proteolipid protein (PLP), and bovine myelin proteolipid protein isoform DM-20 (DM-20)] vary in MW, oligomeric state, and hydrophobicity (see Table 1 and Table S1 of the Supporting Information).

After electrophoresis and staining, we observed that the SDS−PAGE migration behavior of the poly-Leu_{20} standards was distinct from that of commercial blends. As seen in Figure 2, the poly-Leu_{20} polypeptides migrated slower than similarly sized proteins in commercial blends at low MW values, in a manner essentially identical to that of ∼15 kDa commercial proteins, and more rapidly at >15 kDa (Table S2 of the Supporting Information lists proteins in commercial products). These migration trends indicate that poly-Leu_{20} sieves differently on SDS−PAGE than the protein species in the commercial standards, potentially calibrating gels more appropriately for hydrophobic analytes. Calibration curves were accordingly generated for each set of standards by plotting the natural logarithm of protein MW versus the relative migration value (R_f) (Figure 3 and Figure S1 of the Supporting Information).

Apparent MWs of each protein band were then estimated from their measured R_f values using the appropriate line of best fit to each calibration curve. When the proteins were treated as a group, we found that SDS−PAGE calibration with the poly-Leu_{20} product reduces the average deviation of the apparent from actual MWs among the group of membrane proteins tested to ∼7%, compared to the ∼20% deviation from actual values observed with the commercial standards (Table 1). The poly-Leu_{20} standards thus improve SDS−PAGE size estimates for the group of membrane proteins tested to within the 2−7% accuracy

Table 1: Apparent MW Measurements via SDS−PAGE Calibrated with Poly-Leu_{20} and with Various Commercial Standards

		poly-Leu_{20} MW		commercial standards, MW deviation (%)[c]			
protein[a]	MW (kDa)	estimated MW (kDa)[b]	MW deviation (%)[b]	Amersham	PageRuler	Bio-Rad	Mark-12
EmrE 2°	30.4	33.4 ± 2.2	10 ± 7.2	15 ± 1.6	19 ± 1.4	13 ± 2.0	16 ± 4.0
EmrE 1°	15.2	14.3 ± 0.57	5.8 ± 4.0	5.3 ± 1.0	4.3 ± 1.2	3.3 ± 1.6	12 ± 2.4
Hsmr 2°	29.1	28.1 ± 1.2	4.2 ± 3.2	23 ± 1.3	27 ± 3.0	23 ± 3.4	24 ± 2.9
PLP	29.9	29.8 ± 1.1	2.7 ± 2.2	22 ± 0.18	25 ± 0.52	21 ± 1.0	22 ± 2.0
DM-20	26.2	22.9 ± 0.76	13 ± 2.9	25 ± 0.18	27 ± 0.70	24 ± 1.2	27 ± 1.9
	average deviation ± SD[d]		7.1 ± 5.5	18 ± 7.7	20 ± 8.9	17 ± 8.1	21 ± 6.1

[a]Proteins are listed in order of lanes (2−4) as shown in Figure 2. Hsmr and EmrE stoichiometries are as previously reported (*12*, *13*). [b]Mean ± SD of 9−13 independent experiments. MW deviation calculated as the absolute value of (apparent MW − actual MW)/(actual MW) × 100%. [c]Mean ± SD of 3−16 independent experiments. Underlined values are significantly different from the poly-Leu_{20} value ($p < 0.05$ for EmrE; $p < 0.0001$ for Hsmr dimer, PLP, and DM-20). [d]Underlined values are significantly different from the poly-Leu_{20} value ($p < 0.0001$). Deviations among the group of commercial standards were statistically identical ($p > 0.05$).

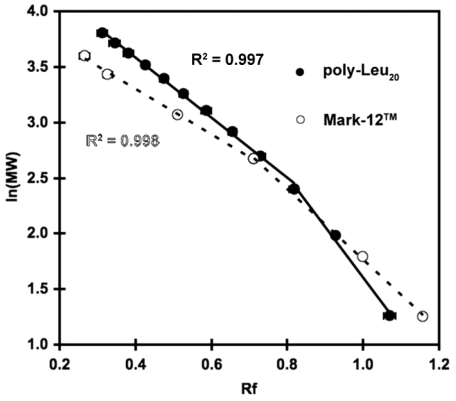

FIGURE 3: Calibration of SDS−PAGE. The natural logarithm of MW of poly-Leu_{20} and Mark-12 polypeptides is plotted vs the mean relative migration value (R_f) ± the standard deviation (SD). R_f was normalized such that smaller proteins display larger values and vice versa. Gels were calibrated using standards in the MW range of 3−45 kDa. Best-fit lines to poly-Leu_{20} (solid) and Mark-12 (dashed) are shown with the correlation coefficient of the line of best fit used to determine apparent MW values. The lines converge at R_f values of ~0.7−0.8 (MW ~ 15 kDa) and diverge above and below this region. The other standards tested are compared to poly-Leu_{20} in Figure S1 of the Supporting Information.

range expected for globular proteins (*6*). We note that standards based on an $n = 12$ Leu peptide synthesized during reagent development did not reach this accuracy level (data not shown). The membrane protein sizing advantage of the poly-Leu_{20} standard was thus hypothesized to arise from the close match of its 20-Leu core to the average length of helical TM segments (*10*).

When the performance of poly-Leu_{20} and the commercial standards was considered on an individual protein level, usage of the hydrophobic standard improved MW estimations of the membrane proteins tested by ~2−10-fold versus at least two sets of commercial standards for all species except monomeric EmrE (Table 1). The equivalent sizing of this monomer by poly-Leu_{20} and commercial standards likely arises from the convergence of their calibration curves at an R_f value of ~0.7−0.8 (MW ~ 15 kDa) (Figure 3 and Figure S1 of the Supporting Information); their divergence at R_f values outside this range explains why the larger proteins are sized more accurately by the hydrophobic standards. We suspect that this gel migration behavior may be characteristic of hydrophobic species, such as the poly-Leu_{20} standards, and membrane protein analytes appear equally subject to this effect. It may therefore be the ability of the poly-Leu_{20} standards to reproduce the SDS−PAGE migration behavior of hydrophobic analytes over their entire calibration range that renders the novel standards capable of providing more accurate membrane protein apparent MW estimates than conventional products. We therefore suggest that, while state-of-the-art reagents remain useful for widespread applications, the increased accuracy provided by hydrophobic standards may facilitate SDS−PAGE experiments involving membrane proteins in which straightforward identification of protein identity and/or stoichiometry is required.

ACKNOWLEDGMENT

We thank Prof. S. Schuldiner (Hebrew University, Jerusalem, Israel) for the generous gift of plasmids for Hsmr and EmrE overexpression.

SUPPORTING INFORMATION AVAILABLE

Full methodological details, Table S1, Table S2, and Figure S1. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Maddy, A. H. (1976) *J. Theor. Biol. 62*, 315–326.
2. Reynolds, J. A., and Tanford, C. (1970) *Proc. Natl. Acad. Sci. U.S.A. 66*, 1002–1007.
3. Choma, C., Gratkowski, H., Lear, J. D., and DeGrado, W. F. (2000) *Nat. Struct. Biol. 7*, 161–166.
4. Walkenhorst, W. F., Merzlyakov, M., Hristova, K., and Wimley, W. C. (2009) *Biochim. Biophys. Acta 1788*, 1321–1331.
5. Rath, A., Glibowicka, M., Nadeau, V. G., Chen, G., and Deber, C. M. (2009) *Proc. Natl. Acad. Sci. U.S.A. 106*, 1760–1765.
6. Goetz, H., Kuschel, M., Wulff, T., Sauber, C., Miller, C., Fisher, S., and Woodward, C. (2004) *J. Biochem. Biophys. Methods 60*, 281–293.
7. Westermeier, R., and Schickle, H. (2009) *Arch. Physiol. Biochem. 115*, 279–285.
8. Schlyer, S., and Horuk, R. (2006) *Drug Discovery Today 11*, 481–493.
9. Verkman, A. S., and Galietta, L. J. (2009) *Nat. Rev. Drug Discovery 8*, 153–171.
10. Ulmschneider, M. B., Sansom, M. S., and Di Nola, A. (2005) *Proteins 59*, 252–265.
11. Hermanson, G. T. (2008) Bioconjugate techniques, 2nd ed., Academic Press, New York.
12. Ninio, S., and Schuldiner, S. (2003) *J. Biol. Chem. 278*, 12000–12005.
13. Chen, Y. J., Pornillos, O., Lieu, S., Ma, C., Chen, A. P., and Chang, G. (2007) *Proc. Natl. Acad. Sci. U.S.A. 104*, 18999–19004.