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A general method for the preparation of mixed micelles of hydrophobic peptides and sodium dodecyl sulphate

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Abstract

A new method is reported for the incorporation of hydrophobic peptides into sodium dodecyl sulphate (SDS) micelles. First, a homogeneous solution of peptide and detergent is obtained by adding the peptide in trifluoroethanol to an equal volume of an aqueous solution of SDS. Upon subsequent addition of excess water, mixed peptide–SDS micelles are formed. Next, all solvent is removed by lyophilization and an appropriate amount of water is added to the dry powder. For various hydrophobic peptides this was shown to yield clear and stable solutions that are highly concentrated and suitable for characterization by spectroscopic techniques.

Key words: Micelle; Two-dimensional NMR; Circular dichroism; Peptide structure; Signal peptide; Gramicidin

1. Introduction

Insight into the structure and function of biological membranes requires knowledge of the structure of the membrane-spanning part of proteins. One potentially powerful technique to obtain detailed structural information of peptides and proteins is two-dimensional NMR spectroscopy (2D NMR). This method requires that the molecules of interest are undergoing rapid isotropic motion. For hydrophobic, membrane-spanning peptides it is therefore most desirable to incorporate them into detergent micelles, which can serve as a membrane-mimicking environment, and which also allow for the rapid isotropic tumbling required for spectroscopy. Sodium dodecyl sulfate (SDS) has been widely used for this purpose [1–3].

Preparation of such mixed peptide/detergent micelles is, however, not trivial. Conventionally, hydrophobic peptides that are insoluble in aqueous solution are added to the micelles from a concentrated organic solution. Homogeneous incorporation of the peptide using this method is difficult since many of the peptide molecules are likely to aggregate before they can be incorporated into the micelles. Also, when the peptide is added as a dry powder, aggregation will interfere with the solubilization of the peptide. In 2D NMR measurements, such aggregational processes will result in line broadening and a loss of signal intensity as the aggregated matter precipitates. Furthermore, since many of the peptide molecules will not be solubilized in the micelles, this will leave a large number of micelles 'empty'.

We now report a simple and general method for sample preparation that results in quantitative and homogeneous incorporation of hydrophobic peptides into SDS micelles and yields optically clear and stable solutions.

2. Materials and methods

2.1. Materials

Sodium dodecyl sulphate (SDS) was obtained from KEBO (Stockholm, Sweden), perdeuterated sodium dodecyl sulphate (SDS-d_{25}) from Cambridge Isotope Laboratories (Woburn, MA), trifluoroacetic acid (TFA) was from Merck (Darmstadt, Germany), 2,2,2-trifluoroethanol (TFE) from Sigma (St. Louis, MO), and 1,1,1,3,3,3-hexafluoro-2-propanol (HFP) from Aldrich (Steinheim, Germany). Water was deionized Millipore Corp. milli-Q water (Bedford, MA, USA). Deuterated water (^2H_2O) was obtained from Dr. Glaser AB (Basel, Switzerland).

Gramicidin A′ (GA′) was purchased from Sigma (St. Louis, MO), as the natural mixture of gramicidins A, B and C [4] consisting of about 80% of gramicidin A, the structure of which is given in Table 1. WAP17, a synthetic peptide designed to form a transmembrane α-helix, was synthesized as decribed below. EPGP, a peptide corresponding to one of the putative membrane-spanning segments of the *E. coli* enzyme phosphatidylglycerophosphate synthase, was synthesized by Dr. Åke Engström (Uppsala, Sweden), as will be described elsewhere. PhoE signal peptide (PhoE SP), corresponding to the signal sequence of the *E. coli* outer membrane protein PhoE, was synthesized by

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Abbreviations: BOC, *N*-α-butyloxycarbonyl; CD, circulair dichroism; 2D-NMR, two-dimensional nuclear magnetic resonance; FMOC, fluorenylmethyloxycarbonyl; HFP, hexafluoroisopropanol; HOBT, 1-hydroxybenzotriazole; OD, optical density; SDS, sodium dodecyl sulphate; TFA, trifluoroacetic acid; TFE, trifluoroethanol.

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Dr. D. Olshevski (San Diego, CA). The synthetic peptides NL10 and L15 were from Bioproducts, Belgium. The amino acid sequences of these different peptides is given in Table 1.

2.2. Synthesis of WAP17

WAP 17 was synthesized by the solid phase method, on an ABI Model 431 Peptide Synthesizer (Applied Biosystems Inc., Foster City, CA). Boc-L-Ala resin (Bachem Bioscience Inc., Philadelphia, PA) was deprotected with 60% TFA in dichloromethane and then neutralized with 10% diisopropylethylamine. Residues L-Trp[16] through L-Trp[2] were coupled as HOBt-esters using Fmoc-amino acid precursors (Bachem Bioscience Inc., Philadelphia, PA) and were deprotected with 20% piperidine in *N*-methylpyrollidinone (ABI, Foster City, CA). The synthesis was completed by coupling previously prepared and recrystallized [5] formyl-L-Ala, also as the HOBt-ester. The peptide was cleaved from the resin using ethanolamine [6] and was removed from the resin by a single, brief rinse with neat TFA.

2.3. General procedure for sample preparation

Unless otherwise stated the following protocol was used. All peptides were first dissolved in TFA (100 mg/ml) and dried under a stream of nitrogen. 5 mM solutions of the peptides were then prepared in TFE. This procedure yielded completely clear solutions for all peptides, except the most hydrophobic L15, which could be dissolved using HFP instead of TFE after TFA treatment. Immediately after preparation, the peptide solution was added to an equal volume of an aqueous solution containing a varying SDS concentration, and water was added to yield a 16:1 ratio of water to TFE by volume. The whole procedure was carried out in glass test tubes at room temperature. The samples were mixed by vortexing for 2 s and lyophilized by rapid freezing in CO_2/acetone, followed by drying overnight under a vacuum of 0.05 mmHg at −80°C. The dry samples were rehydrated with deionized water.

2.4. Turbidity measurements

Unless otherwise stated samples were prepared as described above. For each experiment a total amount of 100 μl of a 5 mM peptide solution in TFE was used, and the samples were rehydrated in 1.7 ml deionized water. The turbidity of different samples was monitored by measurement of the absorbance at 440 nm on a Beckman DU-70 spectrophotometer using a 0.2 cm path length cell at room temperature. To avoid specific absorbance effects all measurements were also carried out at other wavelengths, which yielded similar results.

2.5. ^1H NMR diffusion measurements

Diffusion measurements were carried out as described [7] on a Bruker ACP 250 at 250 MHz at 25°C using 5 mm tubes. For the TFE titration, samples were prepared by diluting 100 μl of a 500 mM SDS solution in $^2H_2O/H_2O$ (9/1, by vol.) with 100 μl of a mixture of TFE and $^2H_2O/H_2O$ (9/1) in the desired volume ratio.

2.6. 2D-NMR measurements

Samples were prepared as described above, using 400 μl of a 5 mM peptide solution in TFE, which was added to 400 μl of a 500 mM solution of perdeuterated SDS in H_2O. After lyophilization the sample was rehydrated in 500 μl H_2O, containing 10% ^2H_2O. 2D NOESY spectra were recorded on a Bruker AMX 500 in the pure-phase absorption mode by application of TPPI [8] with a mixing time of 250 ms, at 40°C. The H_2O (HDO) signal was suppressed by presaturation during the recycle delay and during the mixing time.

2.7. Circular dichroism

Circular dichroism (CD) measurements were carried out on a JASCO 600 spectropolarimeter, using a 0.2 cm pathlength cell, with a 1 nm bandwidth, 0.1 nm resolution, 1 s response time and a scanspeed of 20 nm/min. Spectra of all samples were baseline corrected by using control samples of similarly prepared solutions but in the absence of peptide.

3. Results and discussion

A new method is presented for the preparation of mixed peptide–SDS micelles. The method consists of two steps. In a first step the peptide in TFE is added to a concentrated aqueous solution of SDS, after which excess water is added, resulting in incorporation of the peptide. In a second step the solvent is removed by lyophilization and the samples are rehydrated. We will first characterize both steps in detail and next we will demonstrate the usefulness of this procedure for spectroscopic characterization of hydrophobic peptides.

3.1. Step 1: incorporation of peptides into micelles

Fig. 1 illustrates the first step of the procedure using gramicidin A′ as an example, where the optical density (OD) of the sample at 440 nm is monitored at different stages of sample preparation. In Fig. 1A the sample is prepared according to the more conventional method in which an SDS solution is made or, as in this case, diluted (stage 2) to the desired concentration. The peptide is then added from an organic solution, in this case TFE (stage 3). This immediately results in a turbid sample, as demonstrated by the increased OD, indicating that not all the peptide has been incorporated into the micelles. In Fig. 1B gramicidin is added according to the new method from the same peptide solution in TFE, but now to an equal volume of a concentrated solution of SDS micelles (stage 2). This results in a clear solution, which now stays clear upon subsequent addition of water (stage 3). The

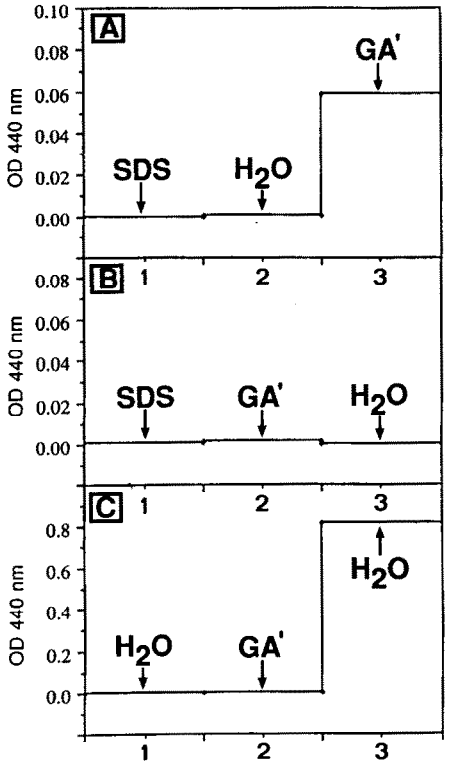

Fig. 1. OD at 440 nm of mixtures of SDS, gramicidin A′ in TFE and H_2O, added together in the following sequences and relative amounts: A: (1) 1 ml 500 mM SDS, (2) +9 ml H_2O, (3) +1 ml 5 mM GA′ in TFE. B: (1) 1 ml 500 mM SDS, (2) +1 ml 5 mM GA′ in TFE, (3) +9 ml H_2O. C: (1) 1 ml H_2O, (2) +1 ml 5 mM GA′ in TFE, (3) +9 ml H_2O.

Table 1
Amino acid sequence of the different peptides

GA:	HCO-VGALAVVVWLWLWLW-NHCH$_2$CH$_2$OH*
WAP$_{17}$:	HCO-AWWAAAAAAAAAAAWWA-NHCH$_2$CH$_2$OH
EPGP:	VEYAGIALFFVAAVLTLWSMLQYLSAAR
PhoE SP:	MKKSTLALVVMGIVASASVQA
NL$_{10}$:	MKKTLLLLLLLLLL
L$_{15}$:	LLLLLLLLLLLLLLL

*Underlined amino acids in the sequence are D-amino acids.

final composition of this sample is exactly the same as that in Fig. 1A. That in this clear solution all peptide must have been incorporated in the micelles is illustrated in Fig. 1C, in which a gramicidin solution is added to an aqueous solution in the absence of SDS micelles (stage 2). When water is added, the sample now immediately becomes highly turbid (note the different OD scale). A behavior very similar to that reported here for gramicidin was observed for all the other peptides in Table 1. This held true even for the very hydrophobic peptide L15, for which HFP was used instead of TFE.

One possible explanation for these results is that SDS micelles break up at the initially high TFE concentrations used, while the peptide molecules are still soluble in this mixture. Upon addition of excess water the peptide then loses its solubility, while at the same time the micelles are re-formed, resulting in the homogeneous incorporation of the peptide. To obtain more insight into this, we investigated the properties of pure SDS micelles in the presence of various amounts of TFE by ^{1}H NMR diffusion techniques. Fig. 2 shows the diffusion rates of the SDS molecules in the presence of increasing amounts of TFE. Only small effects from the solvent are observed below TFE concentrations of 25%, after which the diffusion rate increases linearly with the TFE content. This suggests that the micelles become destabilized at the higher TFE concentrations, resulting in the formation of smaller detergent aggregates. It is probable that this destabilization facilitates homogeneous solubilization of the peptide upon addition of water.

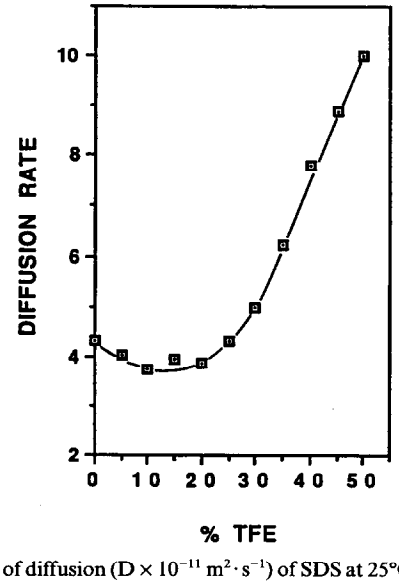

Fig. 2. Rate of diffusion ($D \times 10^{-11}$ m$^2 \cdot$s^{-1}) of SDS at 25°C as a function of the amount of TFE present (volume %). SDS was present as a 250 mM solution in 200 μl total volume of mixtures of D_2O/H_2O (9/1) with TFE.

This method of peptide incorporation yields clear and homogeneous solutions for all peptides tested, and is therefore useful for spectroscopic techniques such as CD. However, these solutions are not suitable for 2D-NMR studies because they are too dilute and contain a relatively large amount of organic solvent. Therefore we investigated whether the procedure could be altered to circumvent (at least partially) these problems. Samples were prepared with the initial amount of TFE reduced, and with the SDS concentration as well as the peptide/detergent ratio varied. Qualitative results, again using gramicidin as an example, are shown in Table 2, which shows that the turbidity of the sample depends on both the peptide/detergent ratio and the initial SDS concentration. At low ratios of 1/250, when a large excess of detergent is present, clear solutions are obtained in the whole range of SDS concentrations measured, but at higher ratios of 1/100 and 1/50, the sample turbidity depends on the actual SDS concentration. The higher the initial SDS concentration, the higher the ratio of gramicidin/SDS that can be used (and therefore the larger the absolute amount of gramicidin), while still obtaining an optically clear sample. Thus, it is possible to adapt the procedure to increase the suitability of such samples for 2D NMR studies. The most ideal situation, however, would be when samples are both highly concentrated and depleted of organic solvent. This situation could be obtained by lyophilization.

Table 2
Turbidity of gramicidin/SDS mixtures

SDS concentration (mM)	Gramicidin/SDS ratio		
	1/250	1/100	1/50
1000	–	–	–*
500	–	±	+
250	–	+	++

Gramicidin/SDS mixtures were prepared by the addition of 10 μl of a gramicidin A′ solution (varying concentration) in TFE to 50 μl of a SDS solution (concentration as indicated) in H_2O. Next, 1 ml H_2O was added to obtain a sufficiently large final volume for OD measurements. Optically clear samples are denoted as –, with + and ++ indicating increasing turbidity.

*Because of the limited solubility of gramicidin in TFE a total of 20 μl of a 50 mM solution of gramicidin A′ was added instead of 10 μl of a 100 mM solution.

3.2. Step 2: lyophilization and rehydration

After the incorporation of different peptides into SDS micelles as described above, a second step was carried out in which the samples were subjected to lyophilization to remove the water and organic solvent, and subsequently were rehydrated in an appropriate amount of water. When mixed at a 1/100 ratio of peptide:detergent, the addition of the first drops of water to the lyophilized powder immediately resulted in a clear and highly concentrated solution for the PhoE signal peptide, WAP17 and EPGP. Apparently, once surrounded by SDS molecules individual peptide molecules remain solubilized in the detergent, and therefore the samples can be easily rehydrated to form clear solutions. Such a lyophilization step may even be of more general use, since recently it was reported for mixtures of alamethicin and SDS that lyophilization from an organic co-solvent also resulted in the formation of mixed micelles [9]. Somewhat surprisingly, ^{1}H NMR diffusion measurements showed no significant decrease in diffusion rate of the SDS micelles upon incorporation of the different peptides (data not shown), suggesting that the micellar size remains approximately the same. The hydrated samples remained clear for at least several months at room temperature, indicating that these peptide-containing micelles are very stable structures. For gramicidin, a clear solution was also obtained under these conditions. However, in this case one important difference was observed: upon addition of water the mixture at first was turbid, but cleared with time (approximately 30 min). The reason for this is not known, but might be related to conformational transitions of the gramicidin molecule in dry and hydrated forms [10].

The turbidity of various peptide/detergent mixtures after rehydration was then measured as a function of the peptide/detergent ratio. A constant amount of peptide in TFE was added to an equal volume of a varying amount of SDS in water. Fig. 3 demonstrates that for three different peptides the turbidity after hydration is highly dependent upon both the peptide and the peptide/SDS ratio. For WAP17 at a 1/25 molar ratio a clear solution was immediately obtained, while for gramicidin A′ a 1/75 ratio appeared sufficient and for the PhoE signal peptide a 1/100 ratio seemed just enough to homogeneously incorporate the peptide. For the two synthetic peptides L15 and NL10 (see Table 1 for sequence) no clear solutions were obtained at a 1/100 ratio. In view of the results described above, it is probable that an even lower peptide/detergent ratio is required to obtain homogeneous incorporation of these extremely hydrophobic peptides. Thus, our studies indicate that it is the peptide/detergent ratio which is most critical for solubilization of the peptide into micelles, rather than the concentration of detergent, as was recently suggested [11].

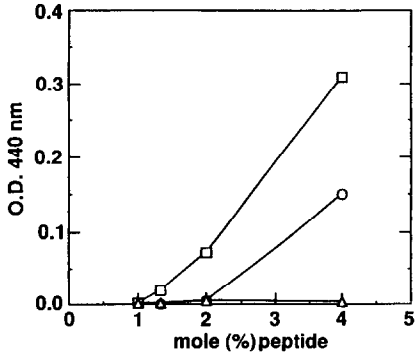

Fig. 3. OD at 440 nm of mixtures of gramicidin A′ (○), PhoE signal peptide (□) and the synthetic peptide WAP17 (△) incorporated into SDS micelles at different ratios of peptide:detergent. For details see text.

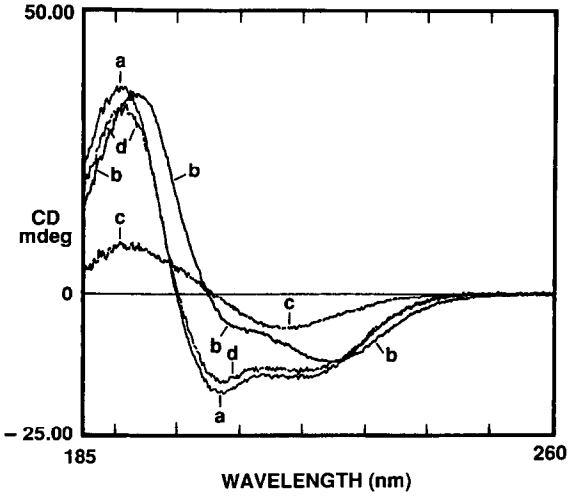

Fig. 4. CD spectra of mixtures of a 10 mM solution of PhoE signal peptide in TFE, with H_2O and with a 1 M solution of SDS in H_2O, added in the following sequence and amounts: (a) 20 μl SDS in H_2O + 20 μl PhoE signal peptide in TFE + 960 μl H_2O, (b) 20 μl SDS in H_2O + 960 μl H_2O + 20 μl PhoE signal peptide in TFE, (c) 20 μl H_2O + 20 μl PhoE signal peptide in TFE + 960 μl H_2O, and (d) similar as in (a) but after lyophilization and rehydration in 1 ml of H_2O.

3.3. Suitability for spectroscopic methods

Fig. 4 shows CD spectra of the PhoE signal peptide in SDS, which demonstrate that this new method is indeed useful for spectroscopic measurements. In the first step of the procedure, the signal peptide in TFE is mixed with an equal volume of a concentrated solution of SDS, followed by dilution with water. This yields a spectrum (Fig. 4, curve a) which is similar to that of the peptide in TFE alone (not shown) and which is typical for the presence of a substantial amount of α-helical structure, with two minima at 208 and 222 nm, a cross-over at about 201 nm and a maximum at 192 nm [12]. Importantly, similar spectra were observed after lyophilization of this sample, according to the second step of the procedure, and subsequent rehydration (Fig. 4, curve d). In contrast, and analogous to the results described in

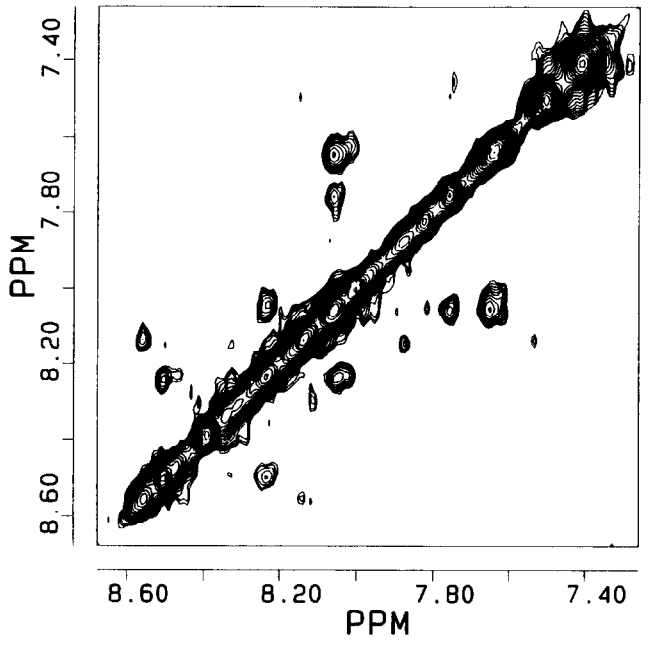

Fig. 5. NH-NH region of 2D NOESY spectra of PhoE signal peptide in SDS-d_{25} micelles at a 1/100 molar ratio and at 40°C. For details see text.

Fig. 1, spectral changes are observed when the peptide is added in the more conventional way from a highly concentrated solution in TFE to a diluted solution of SDS (Fig. 4, curve b). The sample became visibly turbid and exhibited a different line shape, suggesting a decrease in the α-helical structure, and a red shift, indicative of scattering artifacts. When no SDS was present, the CD spectrum of the peptide (Fig. 4, curve c) suggested the formation of β-structure, in agreement with earlier findings [13], and a loss of intensity due to aggregation.

2D-NMR spectra of peptides in an α-helical conformation are expected to have intense N–N NOESY cross-peaks. Fig. 5 shows the NH–NH amide region of a lyophilized and rehydrated sample of the PhoE signal peptide with deuterated SDS. Not only are a number of such cross peaks found, these peaks also are well resolved. Estimated line widths from the 1D spectra are approx. 5 Hz for protons from methyl groups and 10 Hz for the amide protons. For EPGP and WAP17 well resolved spectra with cross peaks in this region were also obtained using deuterated SDS (data not shown). CD measurements of these three samples, after 50-fold dilution in water, revealed spectra typical of large amounts of α-helical structure (data not shown).

4. Concluding remarks

We have developed a new method for the preparation of solvent-depleted mixed peptide–SDS micelles, which yields optically clear, stable and highly concentrated solutions that are suitable for spectroscopic studies. The method appears to be universal, as it worked for very different peptides, including the putative membrane-spanning segment of a transmembrane protein, a signal peptide of the prokaryotic secretion pathway, and a synthetic α-helical membrane-spanning peptide. It may also be universal with respect to the detergent used, as suggested by preliminary experiments using dodecylphosphocholine (data not shown). For these reasons, this new method of sample preparation can be expected to facilitate spectroscopic studies on the structure and dynamics of membrane-bound peptides, and thereby to contribute new insights into the function of membrane proteins.

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