Non-detergent sulphobetaines: a new class of mild solubilization agents for protein purification

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The action of non-detergent sulphobetaines (NDSBs) as new mild agents for protein purification is described. The solubilization effects of non-detergent sulphobetaines are shown in different examples, all obtained under non-denaturing conditions: (1) microsomal proteins extraction; (2) recovery after dialysis of nuclear proteins; (3) reduction of precipitation in isoelectric focusing experiments under non-denaturing conditions; and (4) purification of a membrane-bound serine protease from *Plasmodium falciparum* involved in erythrocyte invasion by malaria merozoites. The absence of a significant denaturation effect induced by NDSBs is demonstrated by tests on β-galactosidase and alkaline phosphatase. A simple NDSB synthesis and some possible explanations of the action of NDSBs are also presented.

INTRODUCTION

A frequent prerequisite of protein purification is their solubilization from tissues, biological membranes or inclusion bodies. Common solubilization agents are salts, detergents, and denaturing agents such as urea or guanidinium chloride.

In most purifications, inorganic salts are added to achieve an isotonic ionic strength. Increasing ionic strength is often an efficient means for the recovery of soluble or peripheral membrane proteins. Under such conditions most proteins are not irreversibly denatured. However, only a low salt concentration is acceptable in samples for electrophoretic processes or ion-exchange chromatography.

In order to extract greater amounts of proteins, it is often necessary to apply more aggressive solutions. These highly concentrated urea or guanidinium chloride solutions result in protein denaturation which may be irreversible.

Purification of integral membrane proteins, or proteins subject to strong hydrophobic interactions, requires the use of detergents (review by Neugebauer, 1990). Unfortunately, the efficiency of a detergent is often correlated with denaturing properties. Renaturation may be difficult since detergents are difficult to remove from purified material.

Detergent sulphobetaines (SBs) have been described. CHAPS (Hjelmeland, 1980) is now extensively used as a mild detergent. More aggressive non-cyclic SBs 3-10, 3-12 and 3-14 (Gonenne and Ernst, 1978; Rabilloud et al., 1990) are very efficient but denaturing (Hjelmeland, 1979; Navarette and Serrano, 1983; Matuo et al., 1985). As yet, highly soluble non-detergent sulphobetaines (NDSBs) have been used only by biochemists in order to counteract electro-endosomosis flows in capillary electrophoresis (Petersen, 1992).

Following a first use of NDSBs in isoelectric focusing (IEF) experiments to screen ionic inter-protein interactions without raising the conductivity, we have found a wider range of applications for NDSBs. In this work, we describe the use of NDSBs as solubilization as well as stabilizing agents in protein purification.

MATERIALS AND METHODS

Materials

Acrylamido buffers, 3-(1-pyridinio)-1-propanesulphonate (NDSB201), sodium ascorbate, standard 1 M HCl solution, CHAPS, SDS as well as chemicals for NDSB preparation were from Fluka (Buchs, Switzerland). Mixed ion-exchange beads (Type TMD 8), β-galactosidase (EC 3.2.1.23) and alkaline phosphatase (EC 3.1.3.1) as well as their respective substrates: *o*-nitrophenyl-β-D-galactopyranoside (ONPGP); and *p*-nitrophenyl phosphate (PNP); were from Sigma (St. Louis, MO, U.S.A.). All other chemicals (analytical grade) were from Merck (Darmstadt, Germany) or Prolabo (Paris, France).

Preparation of SBs

With the exception of the commercial NDSB201 which was dissolved in warm water to give a 2 or 3 M solution and repurified on mixed ion-exchange beads, all other SBs were prepared in the laboratory as follows.

NDSBs with a C_3 bridge between S and N

In a round-bottomed flask, set in a beaker filled with water at 20 °C and equipped with a reflux condenser, a thermometer and side addition, 100 g of propane sultone (0.83 mol) were dissolved in 500 ml of dichloroethane and the amine (0.9 mol) was added slowly under moderate agitation. After overnight reaction at room temperature, the product precipitated. After crystallizing in methanol–water mixtures (typically 98:2% w/w), NDSBs were dissolved in water and the 2–3 M stock solutions were purified on mixed ion-exchange beads. It is important to note that propane sultone is carcinogenic and should be handled with care and that amines are irritant (they also smell rather badly) and should be handled in a fume cupboard. The final yield varied with different NDSBs and was between 50 and 75%; purity is >97% as estimated by t.l.c. It must be pointed out that NDSBs are hygroscopic and should be stored in tightly stoppered flasks.

Abbreviations used: NDSBs, non-detergent sulphobetaines; IEF, isoelectric focusing; PNP, *p*-nitrophenyl phosphate; ONPGP, *o*-nitrophenyl-β-D-galactopyranoside; NDSB201, 3-(1-pyridinio)-1-propanesulphonate; PMSF, phenylmethanesulphonyl fluoride; DTT, dithiothreitol; PI, protease inhibitor.

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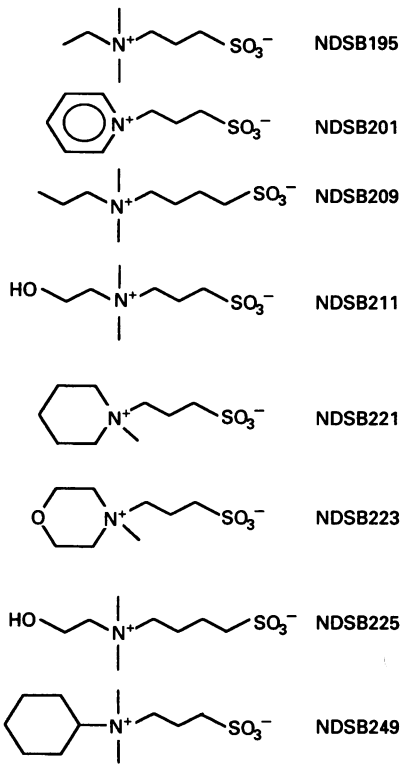

Figure 1 NDSBs used in this work

CH_3 groups are represented by bars connected at one end only.

NDSBs with a C_4 bridge between S and N

In a fume cupboard, the amine (0.9 mol) was dissolved in 100 ml of water–ethanol (50:50) in a 1 litre round-bottomed flask equipped with a reflux condenser and set in a beaker filled with water at 20 °C. Butane sultone (100 g, 0.83 mol) was slowly added under moderate agitation. (Care must be taken to avoid overheating when adding propane sultone to reactive, low boiling-point amines such as trimethyl amine). The reaction mixture was left for 3 days. The mixture was then added to 1 litre of cold acetone. Two phases appeared, with the NDSBs concentrated in the lower one. After elimination of the excess acetone and addition of fresh acetone, the product crystallized. After redissolving in water, NDSBs were purified on a 20 ml column of mixed ion-exchange beads. The products were then recrystallized twice from cold acetone. To allow solubilization in the minimum volume of water, the acetone was thoroughly evaporated after each crystallization step. Although the ease of re-crystallization varied with the various NDSBs, this synthesis is very simple. Yields were in the range of 30–50 % with a purity >97 % as estimated by t.l.c. Formulae are presented in Figure 1.

Microsomal protein extraction

P3-X63-Ag8 plasmacytoma cells were grown in RPMI 1640 medium supplemented with 10 % fetal bovine serum, 10 mM Hepes buffer (pH 7.5), 1 mM pyruvate and 10 μM thioglycerol. The cultures were trace-labelled by incorporation of [^{35}S]-methionine added to the complete culture medium in one of the culture flasks. After labelling for 48 h, the cells were harvested and washed in PBS. The cells were then lysed at 0 °C by incubation in a hypotonic medium [Hepes–NaOH 10 mM (pH 7.5)/1.5 mM $MgCl_2$/1 mM dithiothreitol (DTT), supplemented with the protease inhibitors (PIs), antipain, pepstatin, chymostatin, leupeptin and aprotinin (5 μg/ml of each) and 0.2 mM phenylmethanesulphonyl fluoride (PMSF)] for 30 min, with occasional vortex mixing. The lysate was then passed through a 22-gauge syringe needle to complete lysis and centrifuged at 1000 *g* for 5 min to pellet intact cells, debris and nuclei. The supernatant was collected, centrifuged again under the same conditions and the final supernatant was collected. Aliquot fractions of this supernatant were then ultracentrifuged at 100000 *g* for 1 h to pellet the microsomes.

The proteins from the microsome pellets were then extracted by different solutions, based on buffer E [10 mM Hepes–NaOH (pH 7.5)/2 mM DTT/1 mM EDTA] supplemented with PIs as described above. The solutions were supplemented by one or more of the following additives: 1 % SDS, 0.5 % Triton X-100, 1 M NDSB201, 1 % octyl glucoside, 1 % CHAPS, as described in the Results section.

The microsomes were resuspended at approximately 1 mg/ml of protein in these different solutions and extracted at 0 °C (extracts containing SDS were performed at 20 °C in order to avoid detergent precipitation) for 1 h with occasional agitation. The extracts were centrifuged at 100000 *g* for 1 h to pellet the insoluble material. The supernatants were collected and the amounts of protein extracted were obtained by counting the solubilized radioactivity. The results were expressed relative to the extraction with SDS.

Nuclear protein extraction

BASC6 lymphoma cells were cultured and labelled as described above for the P3-X63-Ag8 cells. The harvested cells were rinsed twice with PBS supplemented with 1 mM EDTA and lysed for 30 min at 0 °C in the nuclear extraction buffer N [10 mM Hepes–NaOH (pH 7.5), 2 mM DTT, 1 mM EDTA, 2 mM EGTA, 0.75 mM spermidine, 0.15 mM spermine, 0.34 M sucrose and 0.125 % Triton X-100], supplemented with the PIs antipain, pepstatin, chymostatin, leupeptin and aprotinin (5 μg/ml of each) and 0.2 mM PMSF. The nuclei were pelleted by centrifugation at 1000 *g* for 5 min and washed twice by resuspension in buffer N and recentrifugation as above. The nuclei were then resuspended at 0 °C in buffer L1 [10 mM Hepes (pH 7.5)/2 mM $MgCl_2$/1 mM DTT]. An equal volume of buffer L2 [10 mM Hepes (pH 7.5)/5 mM EDTA/1 mM DTT/1.6 mM KCl) was added and the contents of the tube immediately mixed by inversion, leading to a very viscous but clear solution. After extraction for 1 h at 0 °C, the DNA and insoluble proteins were pelleted by centrifugation at 200000 *g* for 1 h. The supernatant was then divided into aliquots which were dialysed against solution D1 [10 mM Hepes (pH 7.5)/1 mM DTT/10 mM KCl], supplemented or not with one or more of the following additives: 1 mM urea, NDSB201, NDSB211, as described in the Results section.

After dialysis for 18 h at 4 °C, the dialysates were collected and cleared of insoluble material by centrifugation at 10000 *g* for 10 min. The yield was calculated by comparing the amount of radioactivity in the collected supernatant with the radioactivity in the starting nuclear proteins extract before dialysis.

Extraction of *Plasmodium falciparum* proteins

The Uganda Palo Alto knobby strain (FUP/PAM) of *P. falciparum* was cultured *in vitro* using human A+ red blood cells in RPMI medium supplemented with 10 % human AB+ serum as described (Braun-Breton et al., 1986). The parasite cultures were synchronized (Braun-Breton and Pereira da Silva, 1988)

and ^{35}S metabolic labelling of parasite proteins was carried out by using a 250 μCi/ml Tran-^{35}S Label Kit (ICN) in culture medium for 8 h at 37 °C.

Membrane fractions were prepared as described (Braun-Breton et al., 1986) and finally resuspended in various detergent-containing buffers (see Results section) and incubated for 20 min at room temperature. The suspensions were centrifuged at 15000 *g*, 4 °C for 20 min and the supernatants analysed by SDS/PAGE followed by fluorography. Immunoprecipitations were performed as described (Braun-Breton et al., 1986) with the Hb31cl3 monoclonal antibody, specific to the gp76 protein.

IEF and two-dimensional gel IEF–SDS/PAGE

IEF and IEF–SDS/PAGE (two-dimensional) were carried out as described in Görg et al. (1988). IEF was performed with commercially or laboratory prepared immobilized pH gradient gels. IEF was carried out either under non-detnaturing conditions in the presence of 1 M NDSB or under fully denaturing conditions (8 M urea/50 mM DTT). Silver staining was performed as described by Rabilloud et al. (1994) and the method for colloidal Coomassie Blue staining was from Neuhoff et al. (1988). The authors strongly favour this method to the usual staining with Coomassie R250 dissolved in acetic acid/ethanol, since the sensitivity, as well as the signal-to-noise ratio are clearly increased.

Alkaline phosphatase activity assays

Measurements were performed in a spectrophotometer equipped with cuvettes thermostated at 37 °C. At least triplicate measurements of the absorbance increase at 405 nm upon PNP (1.3 mM) hydrolysis were performed. The enzyme was diluted to a final concentration of 4 μg/ml, incubated overnight in buffer A [50 mM Tris/HCl (pH 8.7)/200 mM NaCl/0.1 mM $ZnCl_2$/0.5 mM $MgCl_2$] containing 0, 0.2 or 1 M NDSB201, and assayed in buffer A in the presence of 0, 0.2 or 1 M NDSB201. The specific activity of the enzyme in buffer A was 76 μmol PNP hydrolysed $min^{-1} \cdot mg^{-1}$.

β-Galactosidase activity in the presence of NDSB201

β-Galactosidase activity assays were performed in a similar way. Hydrolysis of 2 mM ONPGP in buffer B [Hepes/HCl (pH 7.8)/200 mM NaCl/1 mM DTT/1 mM $MgCl_2$] was measured. The commercial suspension (33 mg/ml, specific activity of the enzyme in buffer B: 94 μmol ONPG hydrolysed $min^{-1} \cdot mg^{-1}$) was diluted to a final concentration of 0.2 mg/ml and incubated overnight in buffer B containing 0, 0.2 or 1 M NDSB, then assayed in buffer B in the presence of 0, 0.2 or 1 M NDSB.

β-Galactosidase activity after exposure to pH 4.6

The commercial suspension was diluted to a final concentration of 0.2 mg/ml in 50 mM sodium acetate buffer, pH 4.6, with or without NDSB. After 30 min at 37 °C, the solutions were assayed directly, following a 25-fold dilution in assay buffer B.

RESULTS

The protein solubilization properties of NDSBs were tested on several model systems including membrane- and nuclear-protein extraction and non-denaturing IEF. The absence of significant denaturation induced by NDSBs was checked by enzymic activity assays. These experiments on model systems were then applied to the study of membrane proteins from *P. falciparum*, the major etiological agent of malaria.

Table 1 Labelled membrane-protein extraction yields with buffer containing various additives relative to extraction with 1% SDS taken as 100% yield, corresponding to a protein concentration of 800 μg/ml

Additives	Yield (%)
1% SDS	100
0.5% Triton X-100	64
0.5% Triton X-100/0.5 M NDSB201	74
0.5% Triton X-100/1 M NDSB201	91
1% Octyl glucoside	23
1% Octyl glucoside/0.5 M NDSB201	43
1% Octyl glucoside/1 M NDSB201	65
1% CHAPS	53
1% CHAPS/0.5 M NDSB201	71
1% CHAPS/1 M NDSB201	71

Extraction of microsomal proteins

^{35}S-labelled membrane proteins from murine P3-x63-Ag8 plasmacytoma cells were extracted by various detergents in the presence of varying concentrations of NDSB201. Radiolabelling was preferred to a colorimetric protein assay to avoid possible artefacts due to the presence of different NDSBs in the solutions. The extraction yields were measured as described in the Material and methods section and are expressed relative to the extraction performed with 1% SDS (this is preferred to the measure of a real yield as the counting efficiency is poor for the crude fractions due to the presence of particulate material absorbing the light emission from the radiation quenchers). The results are shown in Table 1.

Whichever detergent was used (Triton, CHAPS or octyl glucoside), adding 0.5 or 1 M NDSB201 clearly increased the protein yield. This effect is particularly marked with octyl glucoside, where recovery is increased 2.8-fold in the presence of NDSB201. In buffers containing Triton X-100, adding 1 M NDSB201 increases the extraction yield to a level close to that obtained with 1% SDS. NDSBs increase the yield obtained by using Triton X-100 or CHAPS by about 30–40%.

The extraction yields varied both with the concentration of NDSB and with the type of detergent used. The effect of NDSB concentration is strong with octyl glucoside, moderate with Triton X-100 and weak for CHAPS.

These quantitative results are confirmed by a qualitative analysis of these extracts by denaturing two-dimensional gel electrophoresis (8 M urea-IEF–SDS/PAGE) (Figure 2). This method (Görg et al., 1988) using immobilized pH gradients has been shown to provide high accuracy and reproducibility.

IEF–SDS/PAGE analysis of extracts obtained with 1% Triton in the presence of 1 M NDSB201 (Figure 2a) show spots that are absent in control IEF–SDS/PAGE from extracts obtained with 1% Triton only (Figure 2b). To avoid differences in staining, the gels were silver-stained together. For the gel showing extracts obtained with 1% Triton only (Figure 2b), the development of the silver stain was slightly longer as shown by a higher background.

These IEF–SDS/PAGE gels show that the solubilization effect of NDSBs is not due to a uniform increase in yield but rather to an increase in the extraction yield of specific proteins.

Nuclear-protein recovery after dialysis following extraction

High salt concentrations are able to extract nuclear proteins efficiently without denaturation (Matuo et al., 1985). However, salts often cannot be tolerated in further steps such as tran-

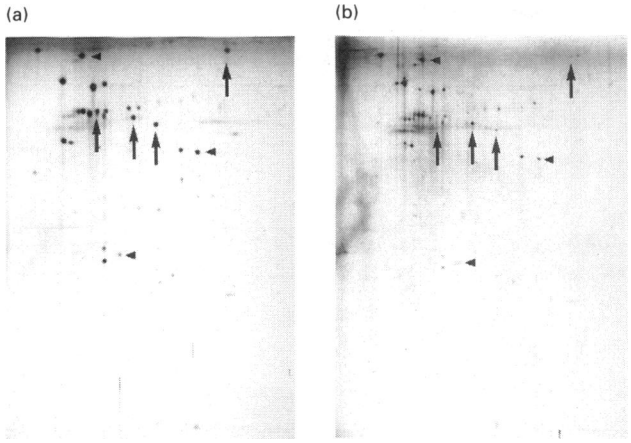

Figure 2 Denaturing two-dimensional gel electrophoresis

Electrophoresis (8 M urea-IEF–SDS/PAGE) from membrane-protein (5 μg) preparation obtained with: 1% Triton X-100 and 1 M NDSB201 (**a**), 1% Triton X-100 (**b**). First dimension: linear immobilized pH gradient, pH 4 (left) to pH 8 (right). Sample load at pH 4. Second dimension: 10% gel for SDS/PAGE. Arrowheads point to patterns present under both conditions showing the high reproducibility of the separation. Some proteins showing increased solubilization with NDSBs are marked with arrows.

Table 2 Labelled nuclear-protein dialysis yields with buffer containing various additives

Additives	Yield (%)
No additives	37
1 M Urea	48
0.25 M NDSB201	60
0.5 M NDSB201	62
1 M NDSB201	95
1 M NDSB211	88

scription *in vitro* (Dignam et al., 1983), DNA binding assays (Strauss and Varshavsky, 1984), or purification by ion-exchange chromatography. Dialysis of solubilized nuclear-protein extracts into lower salt-concentration buffers results in massive precipitation (Dignam et al., 1983; Matuo et al., 1985).

We have successfully applied NDSBs to limit this precipitation induced by transfer of extracted nuclear proteins into lower ionic-strength buffer. The results are presented in Table 2. It is shown that the precipitation is largely suppressed by NDSB201 or NDSB211. However, although NDSBs can prevent the aggregation of nuclear proteins, NDSBs are unable to dissociate the nucleoprotein complexes: an extraction buffer without salt, but containing 1 M NDSB, is hardly superior to an extraction in a low ionic-strength buffer (results not shown). Again, as in the experiments on microsomal proteins extraction, radiolabelling was preferred to a colorimetric protein assay to avoid possible artefacts due to the presence of different NDSBs in the solutions.

Reduction of the precipitation of plasma proteins in non-denaturing IEF

The precipitation of proteins during IEF under native conditions is often a consequence of the negligible ionic strength present during those experiments. Furthermore, the solubility of a protein is generally diminished at pH values close to its isoelectric point.

Since NDSBs remain without net charge over a wide range of pH, NDSBs can be present during electrophoretic separations without raising the conductivity. We have investigated the solubilizing properties of NDSBs in non-denaturing IEF experiments.

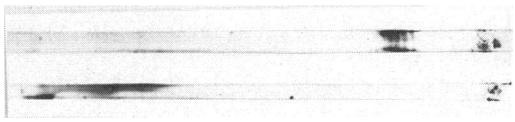

Figure 3 IEF under non-denaturing conditions from fibronectin 110 kDa cell binding fragment (2 μg) in the presence (top) or absence (bottom) of 1 M NDSB201

Linear gradient pH 4 (right) to pH 7 (left). Sample load at pH 7. In the absence of NDSB streaking is observed, in the presence of NDSB the isoforms focus into clearly separated bands.

Figure 3 shows IEF under non-denaturing conditions of a 110 kDa cell-binding fibronectin fragment in the presence (top) or absence (bottom) of 1 M NDSB201. Precipitation that prevents the isoforms from reaching their pI is abolished by NDSB201 where the separation of the various isoforms is clear.

To examine the solubilization by NDSBs in a more complex system, a plasma fraction (polyethylene glycol 4000 9–19% fraction) was used for non-denaturing IEF in the presence or absence of NDSB201. In the presence of NDSB201 (Figure 4a) the number of resolved spots increases (arrows) when compared with a control IEF–SDS/PAGE (Figure 4b).

Although the precipitation is clearly diminished by the addition of NDSB, it must again be pointed out that Figures 4(a) and (b) show non-denaturing IEF separation followed by SDS electrophoresis. The resolution is inevitably lower than when the IEF run is performed under denaturing conditions such as the two-dimensional electrophoresis experiments presented above for microsomal protein extraction, Figures 2(a) and 2(b), where fractions of microsomal proteins were analysed by denaturing IEF with separation in the presence of 8 M urea/50 mM DTT followed by SDS/PAGE.

Absence of significant denaturing effects

The absence of any significant denaturation induced by 1 M NDSB was checked on alkaline phosphatase and β-galactosidase. Their activities can be conveniently measured by spectrophotometric assays. NDSB201 had no influence on the spectral properties of nitrophenols. The changes in absorbance observed were thus only related to enzymic activity.

The absence of any immediate significant influence of the presence of up to 1 M NDSB201 in the assay buffer was shown for both enzymes. In the presence of 1 M NDSB201, the activities of alkaline phosphatase and β-galactosidase were respectively 95 and 80% of the activity of the controls measured in the absence of NDSB201.

Longer incubations before the assay (overnight at 8 °C) in buffer containing 0.2 or 1 M NDSB201 had no effect on the activity of alkaline phosphatase. Surprisingly, for β-galactosidase the activity was increased by 25 and 56% respectively by overnight incubation in 0.2 and 1 M NSDB201. A similar increase in the activity of β-galactosidase was observed after overnight incubation in NDSB195.

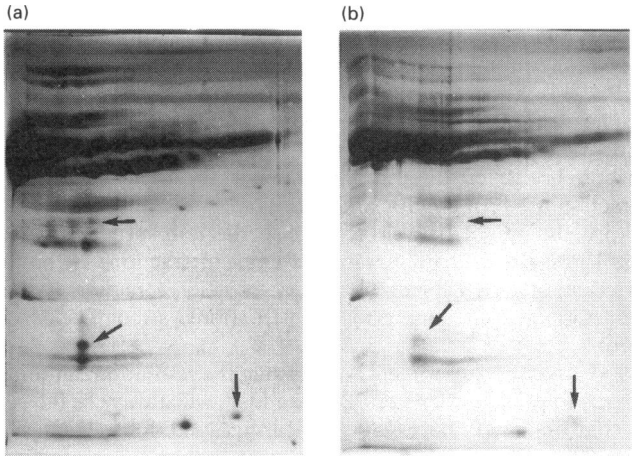

Figure 4 Two-dimensional gel electrophoresis with IEF in the first dimension under non-denaturing conditions (native-IEF–SDS/PAGE) from plasma protein preparation (2 mg total) in the presence (a) or absence (b) of NDSB201

First dimension: native linear immobilized pH gradient, pH 3.5 (right) to pH 5 (left). Sample load at pH 5. Second dimension: 10% linear polyacrylamide gel. Arrows indicate some examples of diminution of streaking or appearance of newly resolved spots upon NDSB use.

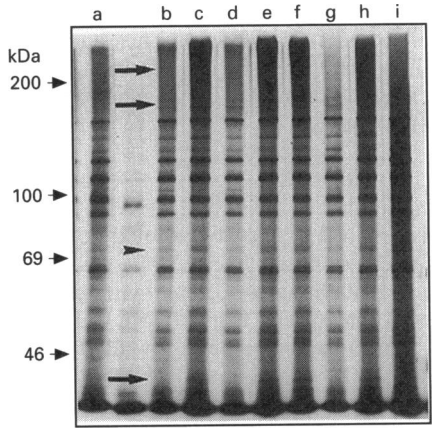

Figure 5 Specific proteins from the parasite, labelled by ^{35}S and solubilized in buffer containing additives, are separated by SDS/PAGE and detected by fluorography

Arrows indicate solubilization of various parasite membrane proteins by buffers containing NDSB 195, 211 and 223. The gp76 is shown by an arrowhead. The additives are: (a) 1% Triton X-100; (b) 1% Triton X-100 + 1 M NDSB181; (c) 1% Triton X-100 + 1 M NDSB195; (d) 1% Triton X-100 + 1 M NDSB209; (e) 1% Triton X-100 + 1 M NDSB211; (f) 1% Triton X-100 + 1 M NDSB223; (g) 1% Triton X-100 + 1 M NDSB225; (h) 1% Triton X-100 + 1 M NDSB (equimolecular mixture of NDSB181–225); and (i) 1% SDS.

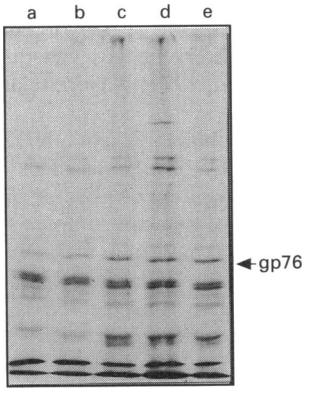

Figure 6 Fluorographic detection of solubilized membrane-bound ^{35}S-labelled gp76, immunoprecipitated using a monoclonal antibody in the absence of NDSB (lane a) or in the presence of NDSB 201, 195, 211 and 223 (lanes b–e)

The gp76 is indicated by an arrowhead. The heat shock protein HSP 72 appears as a doublet immediately below gp76. HSP 72 is a major protein from the parasite, which always contaminates immunoprecipitations and, in this experiment, can be considered as an internal standard.

β-Galactosidase loses activity after incubation at low pH. We have used this property to find out whether any stabilizing property of NDSBs with respect to pH-induced aggregation or denaturation would be observed. The activity of β-galactosidase was determined after exposure to pH 4.6, followed by immediate dilution and activity measurement in buffer B. The substrate hydrolysis rate was higher when the incubation at pH 4.6 was carried out in the presence of an NDSB. NDSB201 or NDSB195 (C_3 bridge between S and N) achieved rates 2.3 and 2.7 times higher respectively than control, as the effect of the NDSB225 (C_4 bridge between S and N) was significantly weaker with an activity rate only 1.5 times higher than the control. Preliminary results obtained with a light-scattering technique showed significant reduction of the aggregation at pH 4.6 in the presence of 1 M NDSB201

Application to the purification of the *P. falciparum* serine protease gp76

Most properties of NDSBs presented above were observed also during the purification of the gp76 *P. falciparum* serine protease. As shown with its mouse homologue (Braun-Breton et al., 1992), gp76 is probably involved in the red blood cell invasion by the parasite. The gp76 protein is synthesized in schizonts as an inactive membrane-bound form possessing a preformed active site (Braun Breton and Pereira da Silva, 1993). In merozoites, the protein is partly solubilized by a parasite glycosylphosphatidylinositol-specific phospholipase C and then exhibits a serine protease activity. Both membrane and soluble gp76 have been purified with non-denaturing IEF as a first step.

As presented in Figure 5, addition of NDSBs to buffers containing Triton X-100, resulted in better solubilization of various parasite membrane proteins (arrows). These include gp76 as well as a high-molecular-mass protein with an M_r of over 200. Total membrane proteins were analysed by SDS/PAGE and specific proteins from the parasite, labelled with ^{35}S, were detected by fluorography (Figure 5). The increase in extraction yield was observed with NDSB195, 211 and 223 but not with NDSB181, 209 and 225.

Specific immunoprecipitation of solubilized membrane-bound gp76, using a monoclonal antibody (Hb31cl3) (Figure 6) shows a significant increase in the recovery of gp76 antigen in the presence of 1 M NDSB. It seems likely that, in the presence of an NDSB, not only the better extraction efficiency of gp76 but also the preservation of its antigenic conformation were responsible for the increase in gp76 recovery. This stabilization has greatly facilitated the purification of the membrane-bound gp76.

NDSB201 (1 M) was also added to the buffers used for the purification of the soluble (i.e. active) form of gp76. Again the

preservation of the antigenic conformation of gp76 by NDSB201 was observed (results not shown). This stabilizing effect was particularly important since the identification of gp76 during the purification steps was achieved by immunoprecipitation with monoclonal antibodies.

DISCUSSION

The NDSBs described in this work should not be confused with the detergent SBs such as: linear SBs (*N*-alkyl-*N*,*N*-dimethylaminopropane-1-sulphonates such as zwittergent 3-16, SB 3-10, 3-12 and 3-14 (Gonenne and Ernst, 1978); CHAPS (Hjelmeland, 1980); and amido sulphobetaines (*N*-alkylamidopropyl-*N*,*N*-dimethylaminoalkyl-1-sulphonate (Rabilloud et al., 1990).

Although detergent SBs and NDSBs are chemically related (having the same functions as well as the same zwitterionic property), NDSBs and detergent SBs differ widely in their physical and biochemical properties. NDSBs do not possess large hydrophobic structures and thus are not amphiphilic and do not form micelles. This is important in experimental considerations since not only can NDSBs be removed by dialysis, but they can diffuse readily in chromatography matrices as well as in polyacrylamide gels for electrophoretic applications. Detergent SBs and NDSBs should function through different mechanisms. With respect to the solubility, the limit of NDSBs is superior to 1 M without micelle formation in comparison with a critical micellar concentration in the millimolar range for CHAPS and as low as 0.03 mM for a detergent SB such as zwittergent 3-16 (Zulauf et al., 1989). Furthermore, with the exception of CHAPS, detergent SBs generally induce protein denaturation (Hjelmeland, 1979; Navarette and Serrano, 1982; Matuo et al., 1985).

Unlike denaturing solubilizing agents, NDSBs are efficient at concentrations where they have little effect on the activity of enzymes such as β-galactosidase or alkaline phosphatase. Those enzymes were chosen since they are complex systems of medium (64 kDa) to high (460 kDa) molecular mass. Both β-galactosidase and alkaline phosphatase are more fragile than enzymes such as ribonuclease alpha, or than some proteases that can undergo complete denaturation/renaturation cycles with a limited loss of activity.

As stated above, there was no perturbation of the colorimetric determinations by NDSBs. When NDSBs were present during the assay, the enzymic activity was only slightly diminished. For β-galactosidase, overnight incubation in NDSB solutions even resulted in an increase of enzymic activity. The most likely explanation of this increased activity after incubation in NDSBs is that a better solubilization of the commercial enzyme suspension in ammonium sulphate occurred when the incubation was performed in the presence of NDSBs. It is well known that β-galactosidase is prone to form multimeric complexes (this was confirmed by our own gel permeation analysis). This is also in line with results obtained with other proteins in the presence of ammonium sulphate. For instance we have been able to crystallize lysozyme with ammonium sulphate only in the presence of NDSBs (L. Vuillard, unpublished work). Furthermore, this increase in activity in the samples containing NDSB is unlikely to be linked to denaturation processes since the activity after overnight incubation in a cold-room in the absence of NDSBs is the same as the activity observed by direct dilution of the stock solution just before the assay. A better solubilization would also explain the results obtained at pH 4.6 if the presence of NDSBs limits the formation of aggregates.

The charge-screening effect and the contribution from the hydrophobic groups of NDSBs are two mechanisms that may help to explain the solubilization properties of NDSBs observed in very different systems such as the murine and *P. falciparum* membrane-protein extractions, the nuclear-protein dialysis and IEF.

The charge-screening effect is based upon the fact that, over a wide pH range, NDSBs bear two opposite charges divided by a carbon bridge creating a dipole. Thus the dipole–charge interaction between NDSBs and the charges present at the surface of proteins might be able to abolish the non-specific ionic or dipole protein–protein interactions. Ionic interactions are bound to occur in systems such as nuclear-protein extraction (strong reduction of ionic strength) and IEF (ionic strength close to zero).

In native IEF experiments on plasma, the resolution, although greatly improved when NDSBs are added, will always be inferior to that obtained under fully denaturing conditions (Hughes et al., 1992). Strong (physiological?) protein–protein interactions will not be abolished by NDSBs. These interactions can prevent proteins from focusing at their isoelectric points. This problem is particularly acute when complex protein mixtures of high concentration, such as plasma, are analysed.

However this charge-screening effect cannot explain by itself the increase in yield observed during membrane-protein extraction experiments. In these cases, it is likely that there is a contribution from the hydrophobic chains linked to the nitrogen in the solubilization process, probably through Van der Waals interactions. This would explain the difference in efficiency of various NDSBs. Results obtained in membrane-protein extraction show that sulphopropyl NDSBs (C_3 bridge between N and S) are more efficient than sulphobutyl NDSBs (C_4 bridge between N and S). In solution, sulphopropyl NDSB might adopt a cyclic conformation (6-atom ring) by an ionic link between N^+ and SO_3^-, resulting in a well-defined hydrocarbon cluster. This cluster might screen some hydrophobic protein–protein interactions. The cyclic conformation is much less favoured with sulphobutyl NDSBs as it would result in a 7-atom ring.

Selecting a precise NDSB for a specific application is difficult at present since there are too few data to define strict guidelines. Nevertheless, it is possible to give some indications. As yet, we have been unable to find any application where C_4 bridge NDSBs would perform better than C_3 bridge NDSBs. We therefore think that, in a new system, C_3 bridge NDSBs are the first choice. Furthermore, most applications in this work were obtained with the C_3 bridge NDSB201. Unfortunately, this chemical possesses a high absorbance in the near u.v. and this will preclude its use in chromatographic systems where protein concentration monitored by measurement of absorbance in the near u.v. is required. Also, NDSB201 is not stable at high pH, which is a serious problem in some electrophoretic systems. Further tests have shown that NDSB195 possesses good solubilizing properties, is easily synthesized and is stable at high pH. As to the spectral properties, NDSB195 does not absorb significantly in the near u.v. and we have checked that in the presence of 1 M NDSB195, there was no significant change in the absorbance of a BSA solution at 280 and 230 nm. We believe that NDSB195 is at present the best choice as a multi-purpose NDSB.

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