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A nondenaturing zwitterionic detergent for membrane biochemistry: Design and synthesis

(solubilization)

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ABSTRACT **The synthesis and evaluation of a new detergent that is a zwitterionic derivative of cholic acid is presented. This detergent combines the useful properties of both the sulfobetaine-type detergents and the bile salt anions. The new detergent proved to be effective at solubilizing membrane proteins in a nondenatured state.**

One of the more important aspects of the purification of membrane proteins is the choice of a suitable detergent. This choice is usually based on the ability to preserve an enzymatic activity or some other native property. In this respect, nonionic detergents (such as Triton X-100 and Lubrol PX) and the bile salts appear to be the reagents of choice (1–3). However, two additional aspects must be considered. The first relates to the artifactual aggregation of proteins in the presence of detergents to form nonspecific protein complexes that have no biological relevance (3). A useful detergent should be capable of breaking such interactions to give maximally disaggregated species in solution. Nonionics are generally less efficient in this respect than are ionic detergents or bile salt anions (1). The second consideration is the extent to which the detergent affects the charge properties of solubilized proteins. Anionic detergents, for example, add substantial amounts of negative charge, which may completely overshadow the charge properties of the native protein. This type of charge alteration profoundly affects the utility of conventional techniques such as ion-exchange chromatography and isoelectric focusing, which depend primarily on charge properties to effect protein separations (4).

A brief survey of existing detergents demonstrates that no single compound that is presently available is adequately nondenaturing, disaggregating, and, at the same time, electrically neutral. The bile salts are both nondenaturing and effective in disaggregating protein but lack the charge neutrality necessary for compatibility with charge fractionation techniques. In contrast, Triton X-100 and other polyethoxy-type nonionic detergents are electrically neutral and nondenaturing but appear not to be efficient at breaking protein–protein interactions (1). *N*-Alkyl sulfobetaines are neutral and efficient at disaggregating protein but unfortunately are strongly denaturing (5).

These considerations suggest that a new detergent with a combination of functional groups providing the most desirable properties of each of the compounds discussed above should prove valuable for the purification of membrane proteins. One obvious possibility is a combination of a bile salt hydrophobic group and a sulfobetaine-type polar group. The synthetic methods to create these types of compounds have been developed (6). This report describes the synthesis and properties of one such compound, a sulfobetaine derivative of cholic acid, and it evaluates its potential utility in membrane protein purification.

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MATERIALS AND METHODS

Tetrahydrofuran (gold label), dimethylformamide (gold label), 1,3-propane sultone (3-hydroxy-1-propanesulfonic acid γ-sultone), triethylamine, 3-dimethylaminopropylamine, and ethyl chloroformate were purchased from Aldrich and used without further purification. Cholic acid (A grade) was purchased from Calbiochem-Behring and dried under high vacuum at 120°C prior to use. Thin-layer chromatography plates of Silica Gel G were purchased from Fisher Scientific.

Microsomes from C57BL/6N mouse livers were prepared as described (5), except that 150 mM KCl/10 mM EDTA, pH 7.25, was used as the homogenization buffer. Measurements of cytochrome *P*-450 content and total protein were also performed as described (5).

For the synthesis of 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, a solution of 40.86 g (0.1 mol) of cholic acid in 500 ml of anhydrous tetrahydrofuran is prepared in a 1-liter round-bottom flask equipped with a drying tube. To this solution is added 13.95 ml (0.1 mol) of anhydrous triethylamine. The flask is gently swirled, 9.56 ml (0.1 mol) of ethyl chloroformate is added, and the flask immediately is placed in an ice bath for 20 min. A voluminous white precipitate should be visible at this point.

To a 1-liter sidearm flask are added 12.54 ml (0.1 mol) of 3-dimethylaminopropylamine and 10 ml of anhydrous tetrahydrofuran. The flask is equipped with a 9-cm Buchner funnel and a no. 1 Whatman filter circle. The contents of the 1-liter flask are then filtered into the sidearm flask. Evolution of carbon dioxide should be visible as the filtrate mixes in the sidearm flask. The round-bottom flask is rinsed with an additional 20 ml of tetrahydrofuran, which subsequently is used to wash the filter cake.

The filtrate then is transferred to a 1-liter round bottom flask, and the tetrahydrofuran is removed by distillation at reduced pressure on a rotary evaporator. The residue is taken up in 500 ml of dichloromethane and transferred to a 2-liter separatory funnel. The organic phase is extracted thoroughly with 200 ml of 3 M sodium hydroxide, and 15 min are allowed for complete phase separation. Small amounts of ethanol (10 ml or less) may be used to break any remaining emulsions. The dichloromethane (bottom phase) is drawn off and dried for 30 min over 50 g of magnesium sulfate. The dried dichloromethane solution is decanted into a 1-liter round-bottom flask. The magnesium sulfate is rinsed with an additional 20 ml of dichloromethane, which is then added to the round bottom flask, and all solvent is subsequently removed at reduced pressure in a rotary evap-

Abbreviation: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.

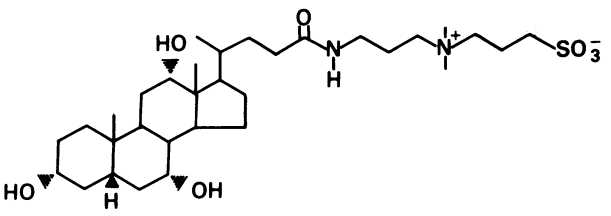

FIG. 1. Chemical structure of 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (suggested trivial name, CHAPS).

orator. Excess water is removed by repeatedly adding 50 ml of toluene/absolute ethanol, 2:1 (vol/vol), to the round-bottom flask, followed by distillation in the rotary evaporator until no cloudiness is observed in the distilled solvent. Removal of all solvents leaves *N*-(3-dimethylaminopropyl)cholamide as a gummy white solid at room temperature.

The gummy white residue from the previous step is dissolved in 300 ml of anhydrous dimethylformamide and transferred to a 1-liter erlenmeyer flask equipped with a ground glass joint and stopper. To this solution is added 12.25 g (0.1 mol) of 1,3-propane sultone, and the flask is stoppered and incubated in a water bath at 60°C for 2 hr. The solution then is allowed to stand overnight at room temperature after which 500 ml of absolute methanol is added. The bulky precipitate is broken up and collected on a Buchner funnel by vacuum filtration, and the filter cake is washed with an additional 200 ml of absolute methanol. The crude product is subsequently triturated in 500 ml of boiling acetone and again collected by vacuum filtration. Thorough drying at room temperature yields 45–50 g of 3-[(3-cholamidopropyl)dimethylammonio)-1-propanesulfonate (75–80% of theoretical yield). The suggested trivial name is CHAPS.

The crude material is judged to be better than 95% pure by thin-layer chromatography on silica gel G in a 95% methanol/5% ammonium hydroxide (vol/vol) solvent system. The product appears as a spot (with R_F 0.32) that can be visualized with iodine. The tertiary amine precursor appears as a spot with $R_F = 0.4$.

Analytically pure material may be obtained by repeated crystallizations at 0°C from absolute methanol, followed by drying under high vacuum at room temperature to a constant weight. The calculated analysis for $C_{32}H_{58}N_2S_1O_7$ after correction for 4.26% water determined by Karl Fischer analysis was: C, 59.85%; H, 9.58%; N, 4.36%; and S, 4.99%. The experimental analysis found: C, 59.85%; H, 9.19%; N, 4.24%; and S, 5.06%.

RESULTS

Fig. 1 shows the chemical structure of CHAPS. The synthesis of this compound was achieved by a combination of well-known techniques for the conjugation of bile salts (7) and by the direct generation of sulfobetaines from tertiary amines and 1,3-propane sultone (6). The overall yield of the isolated product was 75–80% of the theoretical yield and was quite easy to accomplish on a 0.1-mol scale within 1–2 days. The product as isolated was better than 95% pure and was adequate in this state for most applications. The conductivity of a 0.1 M solution of the crude product was approximately 50 μS, indicating a minor salt contaminant. After purification by repeated crystallization from absolute methanol, the conductivity of a 0.1 M solution was reduced to less than 3 μS, and thin-layer chromatography and elemental analysis indicated the compound to be homogeneous.

The absorption spectrum of CHAPS consisted of one major peak at 220 nm due to the secondary amide function and a minor side band with a maximum at 265 nm. The molar absorptivity at 280 nm is approximately 3. This indicates no serious interferences by CHAPS with UV monitoring of proteins at this wavelength.

Data on the effectiveness of CHAPS in solubilizing mouse liver microsomes (Fig. 2) shows that it is nondenaturing with respect to cytochrome *P*-450 at concentrations up to 10 mM. Fig. 2*A* indicates that CHAPS is capable of solubilizing about 70% of the protein of mouse liver microsomes, which can be repelleted in the absence of detergent. Data are given at total protein concentrations of 1, 3, and 5 mg/ml. The sharp break

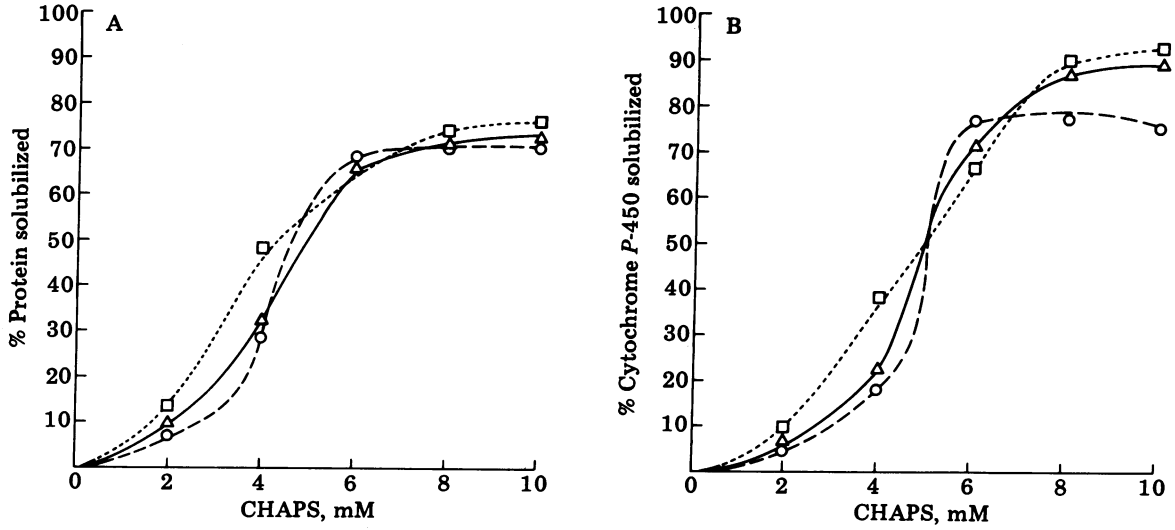

FIG. 2. The solubilization of C57BL/6 mouse liver microsomes by CHAPS. Liver microsomes were prepared by differential centrifugation of tissue homogenized in 150 mM KCl/10 mM EDTA, pH 7.25, at 4°C. For solubilization experiments, the appropriate amount of protein was diluted to give solubilization media with the stated protein and detergent concentrations and with final concentrations of 20% (vol/vol) glycerol and 0.1 M potassium phosphate, pH 7.25. Aliquots (5 ml) were incubated for 30 min at 25°C and then centrifuged at 105,000 × *g* at 25°C for 2 hr. ○, 1 mg/ml; △, 3 mg/ml; □, 5 mg/ml. (*A*) The amount of protein solubilized at various concentrations of CHAPS, expressed as a percentage of the amount of protein that could be recovered in the pellet in the absence of detergent; protein was measured in both the pellet and the supernatant. (*B*) The amount of cytochrome *P*-450 remaining in the supernatant at various detergent combinations; these amounts are expressed as percentages of the total cytochrome *P*-450 content of the intact microsomes. The amount of cytochrome *P*-450 was measured as $A_{450} - A_{490}$ in the reduced vs. reduced plus carbon monoxide difference spectrum.

in the three curves between 4 and 6 mM indicates that the critical micelle concentration of CHAPS is possibly in this region. Fig. 2*B* gives the percentage of the total cytochrome *P*-450 found in the supernatant at each point with the same protein and CHAPS concentrations. The higher protein concentrations yield recoveries of soluble and nondenatured cytochrome P-450 in excess of 90%. CHAPS did not interfere with the Lowry determination of protein at any concentration examined in this study.

DISCUSSION

As a new detergent to be used in the solubilization of membranes, CHAPS combines the useful features of both the bile salts and the *N*-alkyl sulfobetaines. Like the sulfobetaines, CHAPS proves to be somewhat better at solubilizing protein than structurally related carboxylic acid anions (8). Although it is difficult to compare solubilization results from different tissues under different experimental conditions, other studies of the efficiency of solubilization by sodium cholate (9, 10) and our own experience with liver microsomes suggest that CHAPS is a substantially better detergent. The data presented here were obtained with microsomes that were treated with 10 mM EDTA/150 mM KCl to remove extrinsic proteins (11), which account for perhaps 30% of the most easily solubilized protein in these membranes. By this criterion CHAPS behaves more like sodium deoxycholate does in its ability to solubilize total protein, although it is structurally more related to sodium cholate. CHAPS is, however, much more effective at breaking protein–protein interactions than either sodium cholate or Triton X-100 are. Cytochrome *P*-450 is normally highly aggregated in solutions containing either of these detergents, but data to be presented elsewhere indicate that CHAPS disaggregates cytochrome *P*-450 to its monomeric form (unpublished data).

However, the increased capacity of CHAPS to solubilize protein and disaggregate complexes is not gained at the expense of increased denaturing properties. The data presented here indicate that CHAPS is nondenaturing with respect to cytochrome *P*-450 under the conditions employed in these studies. This is in contrast to sodium deoxycholate, which denatures cytochrome *P*-450 under similar conditions. A recent study of the opiate receptor found CHAPS to be the only detergent capable of solubilizing the receptor in a state that showed reversible binding of opioids (12). These are admittedly indirect measures of the physical interactions of CHAPS with these proteins, and the term "denaturing" is only used in its loosest sense. Precise evaluations of the real denaturing power of CHAPS relative to other detergents would need to be performed on purified proteins in which the tertiary structure of the macromolecule could be evaluated at several points on the binding isotherm.

The more important but less obvious advantage of CHAPS as a detergent for solubilizing membranes is its compatibility with charge fractionation techniques. Sulfobetaines, although zwitterionic, behave essentially as nonionic compounds. Specifically, they possess no net charge at any pH between 2 and 12, they exhibit no conductivity or electrophoretic mobility, and they do not bind to ion-exchange resins (13). This should give sulfobetaine-type detergents a tremendous advantage in both ion-exchange chromatography and isoelectric focusing. The utility of *N*-alkyl sulfobetaines in isoelectric focusing has been demonstrated (5, 14), and preliminary experiments with CHAPS indicate no interference with the formation or stability of pH gradients in this technique. Although ion-exchange chromatography in the presence of sulfobetaines has not been specifically investigated thus far, the effectively nonionic behavior of sulfobetaines should make these detergents well-suited for this purpose.

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