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Membrane Proteins

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Proceedings of the 11th FEBS Meeting

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GENERAL INTRODUCTION TO THE PROCEEDINGS

The 11th FEBS Meeting, Copenhagen 1977, was attended by more than 2500 biochemists and their associates. More than 1300 posters, which attracted many spectators and discussants, and about 220 lectures constituted the back-bone of the Meeting. It proved possible to run specialist-sessions on grand topics in five days' symposia as well as colloquia-sessions treating more limited problems.

We hope that the lectures from all six symposia and three of the colloquia published in the Proceedings volumes will be as supportive to our science as they were to the substance of the Meeting.

We are grateful for all the cooperative efforts, in spite of the fact that the work had to be done against deadlines, and also for the support from the Publisher.

Per Schambye

Secretary-General
Professor of Biochemistry
Odense University

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•STATE OF AGGREGATION OF MEMBRANE PROTEINS

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ABSTRACT

Rhodopsin from bovine retinal rods, bacteriorhodopsin from Halobacterium halobium and the calcium-stimulated ATPase from rabbit muscle sarcoplasmic reticulum can be obtained in soluble form in suitable non-denaturing detergents without loss of their native functional or strucutral properties, to the extent that they can be determined. Different detergents, even if closely related, are not equally effective, and results in several detergents have to be compared before one can come to confident conclusions about the state of association in the native membrane. Our studies so far show that bacteriorhodopsin and probably Ca^{++}-ATPase are functionally stable as monomers, which does not imply that they have to be monomers in the native membrane, and bacteriorhodopsin is obviously not, since its native state is crystalline. Rhodopsin is in an oligomeric (probably tetrameric) state in the only detergent studied so far in which bleaching is reversible.

INTRODUCTION

The first step in the characterization of any protein is the determination of its polypeptide chain composition: both water-soluble and membrane-bound proteins frequently exist in nature as complexes consisting of several polypeptide chains. For membrane proteins, detergents have to be used for initial solubilization and purification. Detergents also have to be used during the characterization procedure, whenever it is necessary to have the protein in true solution with a single molecular complex per particle. General principles applying to the use of detergents for both purposes have been described previously (1-3).

This paper is a progress report, describing the work being done in our laboratory to determine the state of association of several important membrane proteins. An important aspect of the work is that different results are often obtained when different detergents are used as solubilizing agents. In the long run this can be a useful tool to probe subtle conformational requirements for the true native state of each protein. Initially, however, one is interested in the question as to which detergent medium provides results most closely reflecting the native membrane-bound state, and our results will be discussed with this question in mind.

CHOICE OF DETERGENT

The effect of detergent on membranous protein/lipid systems depends on how

much detergent is used. At low levels biological activity tends to be retained, but either no solubilization occurs or soluble particles of very large size (effectively membrane fragments) are formed. At higher levels, however, where there are more detergent micelles than protein molecules, small soluble particles are formed containing only a single protein complex per particle. If polypeptide chains remain associated under these conditions, it has to be because an oligomeric state is the thermodynamically stable state, or at least a metastable state prevented from dissociation by kinetic barriers. A very large excess of micelles, coupled with great dilution of the protein, will of course favor dissociation of oligomeric complexes if the associative forces are weak, and one has to keep in mind the fact that effective protein concentrations in membranes are often higher than the concentrations used for characterization in solution. This situation arises rarely in the study of water-soluble proteins. With some exceptions (e.g., hemoglobin), water-soluble proteins usually exist in the native milieu at concentrations below those used for characterization in the laboratory.

It has been understood for some time (4, 5) that detergents can be broadly divided into denaturing and non-denaturing detergents. The former cause gross structural alteration in water-soluble proteins at the levels where one would normally use them for the study of membrane proteins, and therefore are obviously undesirable to use for the solubilization of the latter if one expects to retain the native protein conformation. Non-denaturing detergents usually have little effect on water-soluble proteins and it is reasonable to expect that their principal effect on membrane proteins will be to provide an environment (hydrophobic/hydrophilic interface) that simulates the environment of the native membrane, and that this can be done without significant structural alteration. By this criterion long chain cationic and anionic alkyl derivatives are denaturing detergents and bile salts and nonionic detergents are non-denaturing. Not surprisingly, this simple classification has not proved adequate as experience in the use of these detergents has accumulated, and it is now recognized that not all detergents in the non-denaturing category are equally benign for all membrane proteins. Bile salts, in particular, tend not to be entirely benign, e.g., deoxycholate dissociates the glycoprotein spike complex of the Semliki Forest virus membrane into its constituent glycoproteins (though, unlike sodium dodecyl sulfate, it does so without denaturing the individual proteins), whereas the nonionic detergent Triton X-100 solubilizes the complex in an oligomeric form that is almost certainly the form in which the spikes exist in the viral membrane (6). Deoxycholate (at concentrations required for complete solubilization) irreversibly denatures the Ca^{++}-stimulated ATPase of sarcoplasmic reticulum (7), but for this protein there is also considerable difference in inactivation tendency between different nonionic detergents, even when all of them are in the same general class in that they all have polyoxyethylene chains as hydrophilic moieties (8). In a recent study of mitochondrial cytochrome oxidase, cholate and deoxycholate caused loss of activity, but so did Triton X-100. The nonionic detergent Tween 80, however, solubilized the enzyme in active form and could even be used to reverse the inactivation caused by the bile salts or Triton X-100 (9).

It is clear from these and other studies that the choice of optimal detergent for a given system is still partly a matter of trial and error. The following remarks concerning some specific detergents are offered as possibly helpful in making an initial choice.

Bile Salts.

Structural dissimilarity between bile salts and membrane phospholipids suggests that they are poor candidates for first choice. Data cited above confirm that deoxycholate is not a safe detergent. Cholate, however, has been used less often, and we have found it to be a non-disrupting detergent for rhodopsin (see below).

Amine Oxides.

Dodecyldimethylamine oxide has been used as a solubilizing vehicle for rhodopsin in x-ray and neutron scattering studies. Though formally nonionic, the NO group is extremely polar, and the critical micelle concentration of the detergent is close to that of ionic, denaturing detergents with dodecyl chains. This detergent is therefore potentially a denaturing detergent itself on the basis of a hypothetical explanation for denaturing propensity that we advanced some time ago (5). No studies of the effect of this detergent on water-soluble proteins have been reported, nor have there been comparative studies between this detergent and polyoxyethylene derivatives for any membrane protein. We consider this a risky detergent to use until such studies have been made.

Lysophospholipids.

The mono-acyl phospholipids have all the properties of detergents because they have a single hydrocarbon chain per molecule, and they would seem to be the most promising of all substances for solubilization of membrane proteins because, apart from having only a single acyl chain, they are identical to the diacyl phospholipids of the native membrane. We have not used lysophospholipids in our own work, nor have there been systematic studies in other laboratories.

Simple Polyoxyethylene Ethers.

Detergents formed by an ether linkage between a hydrocarbon chain and a single linear polyoxyethylene chain appear so far to be the most successful detergents for our purposes. The numerous commerical products of this type are all heterogeneous, especially with respect to the number of oxyethylene units. One homogeneous crystallizable compound, dodecyl-octaoxyethyleneglycol (abbreviated $C_{12}E_8$) can be obtained commerically. We have found that variations in either the hydrocarbon moiety or the polyoxyethylene chain are likely to alter the efficacy of polyoxyethylene detergents, and in at least some cases the length of the polyoxyethylene segment appears to be the more important variable. Somewhat surprisingly, there have been no indications so far that Triton X-100, which has a short stubby hydrocarbon moiety (tert-octylphenyl) quite unlike an extended alkyl chain, is dramatically more disruptive than a detergent like $C_{12}E_8$. We have made no systematic comparisons ourselves since we tend to avoid using Triton X-100 because its aromatic chromophore, with an absorption peak close to that of proteins, is an inconvenience.

Tween Series.

In the Tween series of commerical detergents the hydrocarbon chain is linked

to a polyoxyethylene chain by an ester link and the polyoxyethylene chain is part of a branched, bulky complex. The observations on cytochrome oxidase cited earlier suggest that Tween 80 may sometimes be less disruptive than simple polyoxyethylene ethers, but we have found at least one sytem where the reverse is true, i.e., where the bulky head group appears to prevent the detergent from being an effective substitute for phospholipid. It should also be noted that Tween 80, as purchased commerically, is generally contaminated with light-absorbing impurities that are difficult to remove.

REQUIREMENT FOR MEMBRANE PHOSPHOLIPIDS

The question of whether a protein studied in vitro is in exactly the same state as in the physiological milieu is as old as protein chemistry itself. In the case of membrane proteins the question manifests itself in a sense of insecurity whenever membrane proteins are studied in detergent solutions in the complete absence of diacyl phospholipids, an insecurity buttressed by data for a few membrane enzymes that appear to have an absolute requirement for diacyl phospholipids if they are to retain long-term stability of enzyme activity. A general discussion of this topic is beyond the scope of this paper, but it is essential to keep in mind the fact that some membrane proteins are less readily delipidated than others. Use of different detergents, or even use of different solubilization procedures with the same detergent, can than make a difference as to whether or not phospholipid remains associated with the solubilized protein, and this in turn can affect biological activity, the state of association, or both.

RESULTS

Bacteriorhodopsin

Bacteriorhodopsin is especially important in relation to the problem of the retention of native structure in detergents because it is the only membrane protein for which direct structural information, obtained by diffraction methods, is available (10). The protein exists in the purple membrane of Halobacterium halobium in a two-dimensional crystalline array, and electron diffraction studies show that each polypeptide chain (molecular weight 26,000) contains seven side-by-side helical segments, roughly perpendicular to the plane of the membrane, an arrangement leading to a very compact overall shape. The electron density map indicates that the crystalline lattice is made up of individual units each consisting of a cluster of three protein molecules, apparently in close contact with each other.

We have examined the protein in only one detergent, Triton X-100, under conditions where there were about five detergent micelles per polypeptide chain (11). The protein proved to be monomeric, i.e., the trimeric units of the crystalline array were not maintained. Other structural features, reflecting the conformation of individual protein molecules, were the same in the detergent as in the native membrane within the limits of precision of the methods used. Thus circular dichroism measurements indicated an α-helix content of about 70%, in agreement with the percentage of protein represented by the helical segments in the native membrane. Determination of the sedimentation velocity showed that the detergent-solubilized protein is remarkably compact (frictional ratio 1.11 to 1.12), consistent with the

dimensions derived from the electron density map. It should be noted that this latter result is unusual: most intrinsic membrane proteins studied so far are quite asymmetric.

The detergent-solubilized protein also appears to be functionally intact, in that the cycle of photochemical reactions following light-absorption is the same as in the native membrane. The energy-transducing function of bacteriorhodopsin, i.e., its ability to generate a proton gradient across the purple membrane, cannot of course be measured when the protein is in solution. However, fully functional membranes are readily reconstituted after Triton X-100 solubilization.

Rhodopsin

Rhodopsin, the visual receptor protein of the retinal rod cells of vertebrates, contains the same chromophore, retinal, as bacteriorhodopsin, but the retinal is not in the same isomeric state and there is no evidence to suggest that the two proteins are related in terms of amino acid sequence or three-dimensional structure. An important operational difference is that the retinal-protein linkage is very stable in bacteriorhodopsin (which normally functions in the light), whereas the corresponding link in rhodopsin is broken upon exposure to light, and retinal, in the all-trans form, is separated from the protein. Regeneration of native rhodopsin after bleaching requires the addition of retinal in the 11-cis form (in the dark). Purified 11-cis retinal is used in the laboratory, but a mechanism for recycling the all-trans form probably exists in the living cell.

The unique absorption spectrum of retinal in native rhodopsin can be used for two quite different tests for retention of native structure in detergent solutions. One test, retention of the characteristic absorption on storage in the dark, is satisfied even in some denaturing detergents because the conformation in the chromophore binding region is stabilized by the retinal as long as it is in the 11-cis form. The second and more stringent test is to see whether the characteristic spectrum can be restored after bleaching by addition of 11-cis retinal: this is essentially a test of stability of the binding region in the apo-protein, opsin. Both these criteria apply only to that part of the protein molecule that is involved in binding retinal, and it is possible that the structure elsewhere in the molecule can be altered without affecting the binding region.

Table 1 summarizes the available data on retention of native structure in a variety of detergents. The regeneration test is seen to be satisfied in only a few detergents, and a striking aspect of the results is the difference in behavior between cholate and deoxycholate.

We have made molecular weight measurements at 25° in $C_{12}E_8$, where rhodopsin is stable, but opsin is not; and in cholate, where both are stable and regeneration is possible. Rhodopsin is monomeric in $C_{12}E_8$ down to detergent concentrations approaching one micelle per protein molecule. In cholate, on the other hand, the protein is always oligomeric and approaches a limiting molecular weight at high detergent to protein ratios, which probably corresponds to a tetramer of the 40,000 dalton single molecule. (The work is not complete, and an exact limiting molecular weight is not yet established). An important aspect of the results is that partial bleaching in cholate has no effect on the state of aggregation, a result that implies the co-existence

TABLE 1

Conformational Stability of Rhodopsin in Detergent Solutions[a]

Detergent	Retention of native spectrum in the dark	Regenerability after bleaching
Sodium dodecyl sulfate	no	no
Alkyl trimethylammonium bromide	yes (4°)[b]	no
Dodecyldimethylamine oxide	yes	no
Deoxycholate	no	no
Cholate	yes	yes
Digitonin	yes	yes
$C_{12}E_8$[c]	yes	no
Tween 80	yes	yes
Alkyl glucosides	yes	yes

[a] Results from our laboratory and from Refs. 12 and 13.

[b] Slow decay at 25°.

[c] Triton X-100 and other simple alkyl polyoxyethylene ethers behave similarly. Regeneration to a small extent has been reported for Emulphogene B-720 (13).

of opsin and rhodopsin in the same oligomeric particle. Another interesting finding has been to show that the formation of monomers in $C_{12}E_8$ is reversible if the rhodopsin is not bleached, i.e., molecular weight data obtained after replacing $C_{12}E_8$ with cholate are the same as are obtained for rhodopsin originally solubilized in cholate. The oligomeric state in cholate is thus definitely an equilibrium state.

It is tempting in the light of these data to speculate that the tetrameric state is the true native state of rhodopsin, and that opsin formed by bleaching can retain its native structure and ability to recombine with 11-cis retinal when it is part of a tetramer or higher oligomer (as in cholate), but cannot do so when it is monomeric (as in $C_{12}E_8$). However, Hubbard (14) many years ago made a reasonably convincing molecular weight determination in digitonin, another detergent in which regeneration after bleaching is possible. Her result indicated that rhodopsin is monomeric in digitonin. This work clearly needs to be repeated and measurements need to be made in other detergents before a definite conclusion can be reached.

Ca^{++}-ATPase of Sarcoplasmic Reticulum.

The Ca^{++}-stimulated ATPase from rabbit muscle sarcoplasmic reticulum can be reconstituted into vesicles after brief exposure to a variety of detergents, and both ATPase activity and active Ca^{++} transport are regenerated when this is done. However, the enzyme is irreversibly inactivated in most detergents if it is kept in solution for a significant length of time. An earlier report from this laboratory (8) described the first successful solubilization with