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The influence of detergents on the solubility of membrane proteins

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The relationship between the effect of detergents and amphiphiles on protein solubility and their use in crystallization solutions was examined for the reaction center from *Rhodobacter sphaeroides*. Measurement by a centrifugation assay of the solubility of the reaction center as a function of ionic strength revealed dramatic differences in the intrinsic solubility at zero ionic strength in the presence of various detergents and amphiphiles. High protein-solubility values were found for β-octyl glucoside and for lauryldimethylamine-*N*-oxide with heptanetriol. The solubility differences are interpreted in terms of fundamental properties such as the polarity of the detergent molecules. Conditions that resulted in high protein solubility correspond to conditions that have been shown to be successful for crystallization of the reaction center. These results suggest that crystallization is favored for detergents and amphiphiles that optimize the solubility of integral membrane proteins.

1. Introduction

The biochemical properties of integral membrane proteins are intrinsically different to those of water-soluble proteins, as the native membrane surrounding integral membrane proteins must be disrupted by the use of detergent molecules without causing any denaturation. Once membrane proteins are soluble in an aqueous environment, they can be characterized by standard biochemical techniques. However, such studies are often hampered by the generally low amount of protein in cells, difficulties in establishing overexpression systems for integral membrane proteins and their limited solubility. In particular, the major impediment in determining the three-dimensional structures of integral membrane proteins is centered on biochemical properties rather than crystallographic issues (Sowadski, 1996; Bowie, 2000). As a result, our understanding of both the functional and structural properties of many integral membrane proteins is limited owing to the fundamental necessity of establishing proper solubility conditions.

Protein solubility is highly dependent upon the ionic strength (Arakawa & Timasheff, 1985; Riès-Kautt & Ducruix, 1997). At low concentrations, the protein solubility exhibits a 'salting-in' behavior, with the protein solubility increasing with increasing ionic strength. At high ionic strengths, the protein solubility decreases with increased ionic strength in a 'salting-out' behavior. For membrane proteins, the solubility should also be significantly affected by the presence of detergents. The detergent binds to the hydrophobic regions of the protein, forming a belt around the protein. The hydrophilic head group

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of the detergent remaining in contact with the aqueous solution may be charged, uncharged or carry both anionic and cationic charges in ionic, non-ionic and zwitterionic detergents, respectively, resulting in differing distributions of charges on the surface of the protein–detergent complex.

The protein solubility is a key parameter in crystallization, which proceeds by the removal of proteins from solution into a crystalline array. This process is usually achieved by starting with a concentrated protein solution and gradually decreasing the protein solubility with the use of salts or long-chain polymers, causing the protein to leave the solution and form precipitate or crystals (McPherson, 1999). A number of different types of detergents have been used for the solubilization of membrane proteins and in crystallization solutions. The detergents are often supplemented by the inclusion of small amphiphiles that were introduced by Michel (1983) in the crystallization of the photosynthetic reaction center from *Rhodopseudomonas viridis* (Deisenhofer *et al.*, 1985). The mechanism by which amphiphiles act has not been established, although it has been proposed that amphiphiles associate with detergents on the surface of a membrane protein to form a complex that can pack better in a crystal (Michel, 1983). Consistent with this proposal is the observation of a reduction in the radius of the micelles and a decrease in the amount of detergent bound to the reaction center when heptanetriol is included with the detergent lauryldimethylamine-*N*-oxide (LDAO; Timmins *et al.*, 1991; Thiyagarajan & Tiede, 1994; Gast *et al.*, 1994).

The bacterial reaction center serves as a useful model system for the properties of integral membrane proteins. The reaction center from *Rhodobacter sphaeroides* has been well characterized (Feher *et al.*, 1989; Blankenship *et al.*, 1995) and three different crystal forms have been grown using the detergents LDAO and β-octyl glucoside and the amphiphile heptanetriol, yielding structures with resolution limits ranging from 1.9 to 2.8 Å (Allen *et al.*, 1987; Chang *et al.*, 1991; Ermler *et al.*, 1994; Stowell *et al.*, 1997). In this report, we describe a systematic study of the effects of detergents and amphiphiles on the solubility of the reaction center using a relatively simple centrifugation assay. The solubility was determined for a variety of conditions that are commonly employed in crystallization solutions. Both ionic and non-ionic detergents were investigated, either alone or in combination with amphiphiles. Two known amphiphiles, heptanetriol and benzamidine, were included, along with spermine, a molecule that has been useful in the crystallization of tRNA but not yet identified as a possible amphiphile for crystallization of membrane proteins.

2. Materials and methods

2.1. Sample preparation

Reaction centers that were modified by the addition of a polyhistidine tag to the carboxyl terminus of the M subunit were isolated from *R. sphaeroides* and purified as described by Goldsmith & Boxer (1996) with the following modifications. The cells were resuspended in 50 mM NaH_2PO_4 pH 8.0, 150 mM NaCl and 10 mM imidazole and lysed using a French press. The broken cells were centrifuged at 10 000g for 30 min to remove the cell debris. The reaction centers were solubilized by adding 0.65% LDAO and stirring the solution for 30 min at 277 K. This solution was centrifuged at 15 000g for 20 min and the supernatant was loaded onto a column containing Ni–NTA resin (Qiagen). The reaction centers preferentially bound to the resin by the polyhistidine tag and were washed using 50 mM NaH_2PO_4 pH 8.0, 150 mM NaCl, 0.1% LDAO and 20 mM imidazole. After eluting the reaction centers using 50 mM NaH_2PO_4 pH 8.0, 150 mM NaCl, 0.1% LDAO and 250 mM imidazole, the protein was dialyzed against 15 mM Tris–HCl pH 8.0, 0.025% LDAO and 1 mM EDTA. The concentration was determined using optical spectroscopy (ε_{802} = 288 mM^{-1} cm^{-1}; Feher & Okamura, 1978).

Two approaches were utilized to obtain protein samples in the presence of different detergents. One approach was to exchange the detergent by dialysis or sequential dilutions in a buffer containing the desired detergent followed by concentration using either a pressure cell (Amicon) or centrifugation cell (Amicon). Alternatively, the protein was bound to an anion-exchange column, washed in a buffer containing the new detergent and then eluted with salt and dialyzed. The most pure grades of detergents and amphiphiles were purchased from the following sources: LDAO (Fluka), β-octyl glucoside (Calbiochem), dodecyl maltoside (Calbiochem), Triton X-100 (Fluka), cholate (Fluka), deoxycholate (Calbiochem), 1,2,3-heptanetriol (Fluka), benzamidine (Fluka) and spermine (Sigma). The quality of the detergents was checked by standard measurements, such as the critical micelle concentration (Rosenow *et al.*, 2001).

2.2. Determination of solubility

For each combination of detergent and amphiphile, the solubility of the reaction center was determined as a function of ionic strength by a high-speed centrifugation assay. At each ionic strength, solutions were prepared with protein concentrations ranging from very low to saturating values. The solutions were centrifuged in a micro-airfuge (Beckman) at 100 000g for 60 min and the concentration of the protein remaining in solution after centrifugation was measured. Alteration of the centrifugation time from 30 to 90 min did not alter the amount of protein remaining in solution, suggesting that large protein aggregates are being removed from solution.

To determine the solubility value under each condition, the protein at a given initial concentration p_i was compared with the concentration of the protein remaining in the supernatant, p_s. At low protein concentrations, these two values are essentially equal, but as the protein concentration increases, p_s approaches the solubility of the protein. The data for protein concentrations ranging from low values to concentrations near the solubility limit were then fitted (Fig. 1) using the relation

$$p_s = \frac{Sp_i}{S + p_i}, \quad (1)$$

where S is the solubility of the protein. Using this approach, the protein solubility could be determined in the range 0.1–80 mg ml^{-1}. The accuracy was approximately 5%, except for solutions in which the solubility was low (0.1–2 mg ml^{-1}), where the accuracy was approximately 20%. This procedure was repeated at various ionic strengths for each combination of detergent and amphiphile. The use of the micro-airfuge and a microcuvette minimized the amount of protein needed for each measurement.

2.3. Measurement of ionic strength dependence

The solubility S of the reaction center was measured in ammonium sulfate solutions with a range of ionic strengths. These values were fitted with a general solubility dependence that describes both the salting-in and salting-out region, given by the Cohn–Green expression (Riès-Kautt & Ducruix, 1997),

$$\log S = \log S_0 + k_i' I^{1/2} - k_o' I, \qquad (2)$$

where S_0 is the solubility of the protein at zero ionic strength, k_i' is the salting-in constant, k_o' is the salting-out constant and I is the ionic strength. For the conditions where the solubility at low ionic strength was found to be greater than could be measured accurately, the parameter S_0 was constrained to be at least 100 mg ml^{-1} and the fit was determined for data in the high ionic strength region only.

3. Results

3.1. Reaction-center solubility dependence on ionic strength

The solubility of the reaction center in LDAO, β-octyl glucoside and cholate was measured as a function of ionic strength (Fig. 2). The reaction center in LDAO without the presence of any small amphiphile exhibited the salting-in and salting-out regions seen for water-soluble proteins. The reaction center was significantly more soluble at all values of ionic strength in the presence of the detergent β-octyl glucoside, with a solubility exceeding 100 mg ml^{-1} at low ionic strength. In cholate, the protein was more soluble than in LDAO at low ionic strength but had a solubility comparable to that in LDAO in the ionic strength range 2–4. The presence of the amphiphile heptanetriol in LDAO resulted in an approximately 100-fold increase in the solubility of the reaction center compared with LDAO alone. This large change in solubility was not observed with the addition of heptanetriol to cholate or β-octyl glucoside. All three parameters in (2) were determined accurately for the solubility of the reaction center in LDAO alone (Table 1). For the other conditions, the solubility at low ionic strength was greater than 100 mg ml^{-1} and could not be measured, so the parameter S_0 was constrained to be at least this value. Fits of the solubility behavior of the reaction center in β-octyl glucoside or cholate

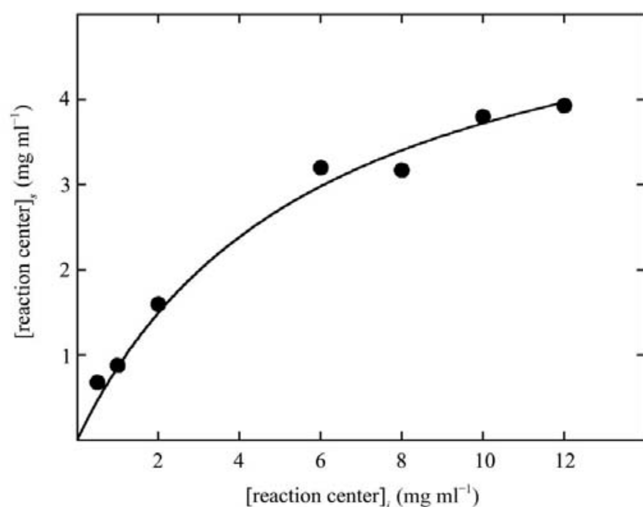

Figure 1
The amount of reaction center present in the supernatant after centrifugation, [reaction center]$_s$, as a function of the initial protein concentration, [reaction center]$_i$. The amount in solution is identical to the initial amount at low concentrations but decreases relative to the initial value as the solubility limit is reached. The solubility is determined by a least-squares fit using (1). The data shown are for 0.025% LDAO and an ionic strength of 0.15.

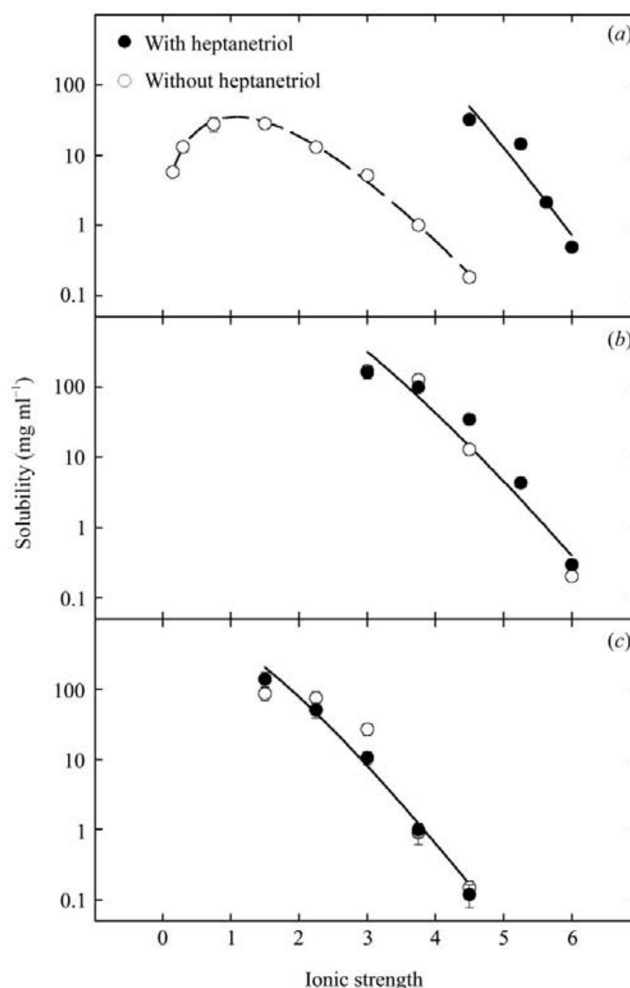

Figure 2
The dependence of the reaction-center solubility on the ionic strength for three detergents, (a) LDAO, (b) β-octyl glucoside and (c) cholate, both without and in the presence of the amphiphile heptanetriol. The curves are fits of the data using (2).

Table 1
Summary of parameters describing the ionic strength dependence of the solubility of reaction centers in different detergents and amphiphiles.

The parameters were determined by a least-squares analysis of the ionic strength dependence of the solubility data using (2).

Detergent and amphiphile	S_0	k'_i	k'_o
LDAO	0.7 ± 0.1	3.9 ± 0.2	1.9 ± 0.1
LDAO with 3% heptanetriol	100†	4.6‡	2.2‡
β-Octyl glucoside§	100†	3.4‡	1.8‡
Cholate§	100†	1.8‡	1.7‡

† The solubility at low ionic strength was greater than 100 mg ml^{-1} and could not be measured accurately, so the parameter was constrained to be at least this value. ‡ The parameters have an estimated error of 10%. § Fits of the solubility behavior of the reaction center in β-octyl glucoside or cholate yielded the same result for solutions with and without heptanetriol. These parameters are from least-squares fits performed using both sets of data.

Table 2
Solubility of reaction centers in different detergents and amphiphiles.

Solubility† (mg ml $^{-1}$)				
Detergent	No amphiphile	Heptanetriol	Benzamidine	Spermine
β-Octyl glucoside	13.0‡	34.5‡	18.0	17.0
LDAO	0.18	32.6‡	1.7	1.0
Cholate	0.15	0.12	0.52	5.91
Dodecyl maltoside	<0.1	9.6	<0.1	<0.1
Triton X-100	<0.1	<0.1	<0.1	<0.1

† Solubility values determined as shown in Fig. 1 at an ionic strength of 4.5 in ammonium sulfate with detergent concentrations of 0.025% LDAO, 0.8% β-octyl glucoside, 0.01% dodecyl maltoside, 0.05% Triton X-100 and 0.3% cholate and amphiphile concentrations of 3% 1,2,3-heptanetriol, 3.0% benzamidine and 50 m*M* spermine. ‡ Conditions successful in crystallization of reaction centers (Allen *et al.*, 1987; Chang *et al.*, 1991; Ermler *et al.*, 1994; Stowell *et al.*, 1997).

yielded the same result for solutions with and without heptanetriol.

3.2. Reaction-center solubility in different detergents and amphiphiles

The solubility of the reaction center in solutions with five types of detergents and three types of amphiphiles was measured at an ionic strength of 4.5 using high-speed centrifugation (Table 2). The tests consistently indicated that the highest overall protein solubility was found for solutions with β-octyl glucoside. A comparably high solubility was found for LDAO with heptanetriol and, in general, the addition of heptanetriol increased the protein solubility. While solutions with cholate, dodecyl maltoside and Triton X-100 yielded relatively low solubility values, in specific cases the addition of amphiphiles increased the solubility significantly. For example, the addition of spermine significantly increased the reaction-center solubility in cholate.

The comparative protein solubilities were not susceptible to variations in the sample preparation and measurements. To test whether the protein solubility was limited by the detergent concentration, the reaction-center solubility was determined for a range of one to four times the critical micelle concentration of the detergent. No significant differences in the relative protein solubility for the various detergent and amphiphile combinations was observed when the detergent concentration was increased. For example, when the LDAO concentration was varied between 0.025 and 0.1%, the solubility remained low at less than 1 mg ml^{-1} when no amphiphile was present, was less than 2 mg ml^{-1} in the presence of the amphiphiles benzamidine and spermine and stayed within 5% of the high value of 33 mg ml^{-1} at 0.025% in the presence of heptanetriol. As expected, significantly decreasing the LDAO concentration to values far below the critical micelle concentration resulted in a decrease in the protein solubility. Within the error of the measurement, no differences were observed if a detergent was exchanged by dialysis or column chromatography after protein isolation. Preliminary measurements for other salts such as sodium chloride were also consistent with the same relative dependences of the protein solubility for different detergents and amphiphiles.

Most previously reported measurements of the solubility of proteins have involved the determination of the protein concentration from crystallization solutions rather than from a solution with an amorphous phase as performed here. To directly compare the two approaches, a series of solubility and crystallization tests were performed using lysozyme (hen egg-white; Sigma). The solubility was determined as a function of ionic strength, which was varied from 2.5 to 4, by measuring the amount of protein remaining in a crystallization solution using optical spectroscopy (ε_{280} = 26.4 M^{-1} cm^{-1}). These solubility values (data not shown) were found to be identical to those previously reported (Ataka & Tanaka, 1986). The same dependence of solubility on ionic strength was determined using the centrifugation approach, except that an approximately 50% greater solubility value for all ionic strengths was obtained (data not shown), probably reflecting the different experimental conditions, such as the temperature.

4. Discussion

In this study, we determined the solubility of an integral membrane protein, the bacterial reaction center, in various detergent and amphiphile combinations by measuring the amount of protein remaining in solution after high-speed centrifugation. This quantitative assessment allows modeling of the solubility and demonstrates that the prominent effect of detergents and amphiphiles on membrane-protein solubility can be used to rank the usefulness of the many possible detergent/amphiphile combinations.

4.1. Reaction-center solubility dependence on ionic strength

To gain a better understanding of the properties of the reaction center in crystallization solutions, the solubility of the reaction center was measured as a function of ionic strength for three detergents, LDAO, β-octyl glucoside and cholate (Fig. 2). The data were analyzed with expressions that have been used to relate the solubility and crystallization of water-soluble proteins such as lysozyme (Ducruix & Riès-Kautt, 1990). As described by (2), at low ionic strength the solubility

is governed by k_i', the salting-in constant, and at high ionic strength it is governed by k_o', the salting-out constant. These parameters are controlled by distinct dominant interactions in the low and high ionic strength regimes and the relative values obtained can be interpreted in terms of physical aspects of the protein–detergent complex as described below.

At low ionic strengths, the solubility of a protein is primarily determined by electrostatic interactions between proteins and salt ions. The increase in solubility with increasing ionic strength is described by the Debye–Hückel theory (Tanford, 1967) with the expression

$$\ln\left(\frac{S}{S_0}\right) = \frac{Z_p^2 e^2 N_A \kappa}{2DRT(1+\kappa a)}, \quad (3)$$

where S is the solubility of the protein, S_0 is the solubility at zero ionic strength, Z_p is the net charge of the protein, e is the electronic charge, N_A is Avogadro's number, D is the dielectric constant, R is the universal gas constant, T is the temperature, a is the sum of the radii of the central and mobile ions in solution and the Debye–Hückel parameter κ is defined as

$$\kappa = \left(\frac{8\pi N_A e^2}{1000 D k_B T}\right)^{1/2} I^{1/2}, \quad (4)$$

where k_B is the Boltzman constant and I is the ionic strength. Screening of the ionic atmosphere around the protein decreases the electrostatic free energy of the protein, which results in a decrease in its activity and an increase in its solubility. This behavior explains why the solubility of a protein is at a minimum at the isoelectric point, where the net charge on the protein is zero.

The detergent bound to the reaction center will primarily alter the electrostatic interactions through the charge Z_p of the protein–detergent complex (3). For example, the high protein solubility observed for the protein–detergent complex with the negatively charged detergent cholate at low ionic strength (Fig. 2) can be attributed to an electrostatic repulsion. The addition of heptanetriol to solutions with LDAO probably results in the binding of heptanetriol, forming a complex with more non-polar character than for LDAO alone, with a consequent increase in the solubility, as is observed. Also influencing the solubility are other factors, including the hydrophobicity of the bound detergent, which is related to the surface exposure and length of the hydrocarbon chains. The relative importance for the solubility of each of these factors will vary among proteins.

At high ionic strength the electrostatic interactions are screened by the presence of the salt and the protein solubility is determined by interactions between the protein and the water molecules. The decrease in solubility with increasing ionic strength has been modeled by Kirkwood (Arakawa & Timasheff, 1985; Kirkwood, 1943) as

$$\log S \propto -(b^3/a)\alpha(b/a)I, \quad (5)$$

where b is the radius of the protein molecule, a is the sum of the radii of the central and mobile ions in solution and $\alpha(b/a)$ is a function given by Kirkwood. The solubility is dependent on the geometry of the protein molecule as electrostatic interactions are screened and the determining factor is the competition between the salt ions and the protein for water molecules. When water molecules become scarce, proteins begin to associate with one another, forming aggregates that are removed from solution. This dependence, given by the parameter k_o' in (2), was found to be similar for the data from each set of conditions. This is consistent with the idea that this parameter is largely determined by the overall shape of the protein–micelle complex, which should be approximately the same for each detergent.

4.2. Impact of detergents and amphiphiles on protein solubility

It has long been recognized that detergents play a fundamental role in the biochemical properties of integral membrane proteins (Helenius & Simons, 1975; Tanford & Reynolds, 1976). The results reported here suggest that one of the primary effects of detergents and amphiphiles is to increase the intrinsic solubility of the protein [S_0 in (2)] with certain combinations of detergents and amphiphiles resulting in significantly increased solubility.

Because the most favorable conditions for crystallization generally make use of protein concentrations of 10 mg ml^{-1} or higher, relatively high solubility values should also be associated with conditions used to crystallize membrane proteins. The detergents and detergent/amphiphile combinations that resulted in a high reaction-center solubility in the high-speed centrifugation assay included β-octyl glucoside alone, β-octyl glucoside with heptanetriol and LDAO with heptanetriol, which are the same combinations that have been successful in growing the three crystal forms of the reaction center (Allen *et al.*, 1987; Chang *et al.*, 1991; Ermler *et al.*, 1994; Stowell *et al.*, 1997). The high solubility found for β-octyl glucoside is also reflected in its use in obtaining structures for a number of other membrane proteins, such as the cytochrome bc_1 complex (Zhang *et al.*, 1998) and the light-harvesting II complex from *Rhodopseudomonas acidophila* in combination with benzamidine (McDermott *et al.*, 1995). In addition to its use in the crystallization of reaction centers, the combination of LDAO with heptanetriol has been effective in crystallizing the FepA β-barrel (Buchanan *et al.*, 1999) and the light-harvesting II complex from *Rhodospirillum molischianum* (Koepke *et al.*, 1996).

Although many factors have an impact on the crystallization process and crystals can grow from solutions containing low concentrations of protein, the results indicate that solubility tests can be a highly effective means of identifying conditions that are likely to produce crystals. For the pigment–protein complex under study, the protein concentrations could be accurately and easily measured from the optical absorption of the pigments. In general, the protein concentration can also be measured for proteins without pigments by measuring the absorption at 280 nm. The results suggest that detergent/amphiphile combinations may be useful owing to the intrinsic properties of these surfactant molecules.

Thus, provided they do not readily denature the protein, specific combinations may result in high solubilities for many different integral membrane proteins. Protein solubility is also dependent upon the specific properties of any precipitating agents. While the solubility measurements were performed in ammonium sulfate, other precipitating agents such as polyethylene glycol are often used for crystallization. The dependence of the solubility of the reaction center upon polyethylene glycol concentration has been reported based upon measurements of the amount of protein remaining in solutions after orthorhombic crystals had grown (Odahara *et al.*, 1994; Gaucher *et al.*, 1997). Preliminary measurements of the solubility in solutions containing polyethylene glycol performed using the centrifugation approach agree with the results of Gaucher *et al.* (1997) and the expected inverse relationship between the log of the solubility and the concentration of polyethylene glycol was observed. Thus, the relative effectiveness of specific combinations of detergents and amphiphiles in establishing high protein solubility is probably independent of the choice of precipitant. However, the use of polyethylene glycol rather than a salt will affect other solution properties, such as phase separation. Therefore while the solubility assay can be used to pre-screen effective detergent/amphiphile combinations, the solubility effect must be balanced by other factors in crystallization.

5. Conclusions

These results suggest that detergents should be favored if they result in a high protein concentration in crystallization solutions and that certain detergent/amphiphile combinations are especially effective, such as heptanetriol with LDAO or spermine with charged detergents such as deoxycholate. For crystallography experiments, the choice of detergent/amphiphile combinations can be further reduced by combining the solubility tests with light-scattering measurements that can provide indications of favorable interactions through the second virial coefficient (George *et al.*, 1997; Hitscherich *et al.*, 2000). Together, these initial characterizations can be used to dramatically reduce the total number of conditions that must be screened and alleviate the requirement for large quantities of protein.

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