Dissolution of Proteins in Organic Solvents using Macrocyclic Polyethers: Association Constants of a Cytochrome c–[1,2-$^{14}C_2$]-18-Crown-6 Complex in Methanol

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Crown ethers and cryptands have been found to be selective complexing agents for a number of proteins, allowing their dissolution in non-aqueous solvents; by using ^{14}C-radiolabelled 18-crown-6, an association constant of *ca.* 1.0 M^{-1} at 299 K for a cytochrome c–[1,2-$^{14}C_2$]-18-crown-6 complex was determined in methanol.

The complex formation between crown ethers (CE) and neutral polar molecules has been examined[1—3] for a number of well defined stoicheiometric complexes. Specific complexation of cations by crown ethers and cryptands is known to facilitate the solubility of simple salts in organic solvents and to provide non-solvated anions by increasing the dissociation of ion pairs. We report here our recent discovery that some proteins can be made soluble in non-aqueous solvents by weak complexation with crown ethers and cryptands. Various other phase transfer reagents (*e.g.* tetra-alkylammonium salts, polyethylene glycols, polyethylene glycol ethers) do not fulfil this function.

Table 1. Proteins that dissolve in methanol using crown ethers and a cryptand at 25 °C.

Protein[a]	Iso-electric point, pH_I (ref. 6)	Molar ratio of protein : crown ether				
		12-crown-4	15-crown-5	18-crown-6	21-crown-7	cryptand-[2.2.2]
Bovine insulin (A and B)	5.72		insoluble	1 : 1200		1 : 120
Cytochrome c	9.3	1 : 1200	1 : 1200	1 : 120—300	1 : 800	1 : 120
Bovine serum	5.2	insoluble		1 : 430	1 : 6000	1 : 300
Lysozyme	11.0			1 : 150		
Myoglobin	7.5		insoluble	1 : 400		1 : 200
α-Chymotrypsin	8.38		insoluble	insoluble		insoluble

[a] Protein concentration was 0.4 mM. Small portions of CE (*ca.* 2 mg) were added to a suspension of protein in methanol with stirring, until suspension dissolved.

Table 1 lists the molar ratios of macrocycle : protein required for dissolution in methanol at 25 °C. Although simple amino acids and small peptides (*e.g.* glutathione) dissolve in methanol containing relatively low concentrations of crown ether (amino acid : crown ether *ca.* 1 : 3—6), higher concentrations are necessary for protein solubilisation (protein : CE, 1 : >100). A low value of the overall association constants (K_s) would account for the large excess of crown ether needed to shift the equilibrium in favour of complexation (equation 1).

$$n\text{CE} + \text{Pr} \xrightarrow{K_s} [\text{CE}_n\text{Pr}] \quad (1)$$

CE = crown ether, cryptand
Pr = Protein, enzyme
n = number of binding sites

Protein–crown ether complexation appears to be dependent upon a number of factors; the cavity size of the macrocycle is important and both 18-crown-6 and cryptand[2.2.2] (cavity size 2.6—3.2 Å) provide a better 'fit' for proteins than either smaller or larger ring macrocycles. Complexation is also dependent upon solvent. Solvents that dissolve cytochrome c with crown ether (*e.g.* methanol, ethanol, dimethylsulphoxide) have good hydrogen bonding capacity and high dielectric constant. Lastly, protein primary, secondary, and tertiary structure seem to be related to the dissolution process. From the data so far, proteins containing both higher proportions of α-helix and high isoelectric point values pH_I are easier to dissolve than those containing a predominance of β-pleated sheet structures and/or low pH_I values.

To our knowledge there are no previous reports on association constants between crown ethers and proteins. Measurements of association constants, K_s (equation 1), were carried out using ^{14}C-radiolabelled [1,2-$^{14}C_2$]-18-crown-6 by equilibrium dialysis techniques. The radiosynthesis of [1,2-$^{14}C_2$]-18-crown-6 was carried out starting with $^{14}C_2$-oxalic acid (specific activity 3.3 GBq/mmol).[4,5]

T.l.c. (SiO_2 using 20% methanol–diethyl ether, R_f for 18-crown-6 0.15) and radioscanning showed the product to be radiochemically pure; the chemical purity of [1,2-$^{14}C_2$]-18-crown-6 was 74% as determined by g.c. using 10% Carbowax 20 M at 230 °C, [1,2-$^{14}C_2$]-18-crown-6 retention time, r.t., 12.2 min; impurities had r.t. 10.7 min (20%) and r.t. 4.8 min (6%) respectively and these did not contain any ^{14}C label; ^{1}H n.m.r. spectroscopy 60 MHz in CD_3OD; $-OCH_2$ δ 3.63. Equilibrium dialysis techniques were employed for binding studies. Protein was mixed with [1,2-$^{14}C_2$]-18-crown-6 in methanol at a specified temperature and concentration (Table 2) and introduced above a membrane (exclusion limit 5 000 Daltons). After centrifugation (2 000 g, 2 min) the difference in counts per unit volume between the upper and lower layers represents the radiolabel which is bound to the protein, [CE.Pr].†

Table 2. Measurements of K_s of cytochrome c–[1,2-$^{14}C_2$]-18-crown-6 in methanol.

Molar ratio crown ether : protein	Temperature (K)	K_s(M^{-1})[a]
50 : 1	299	1.2
130 : 1	299	0.84
165 : 1	299	0.94
138 : 1	283	3.0
171 : 1	276	4.7
130 : 1	288	2.93
130 : 1 (maleylcytochrome[b])	288	1.6

[a] $K_s = \dfrac{[\text{CE.Pr}]}{([\text{CE}]_0 - [\text{CE.Pr}])([\text{Pr}]_0 - [\text{CE.Pr}])}$

where [CE.Pr] is the concentration of the crown ether–cytochrome c complex calculated as shown in footnote † from Δ; $[CE]_0$ and $[Pr]_0$ are the initial concentrations of crown ether and protein respectively. The specific activity of [1,2-$^{14}C_2$]-18-crown-6 was determined by g.c./liquid scintillation counting using a calibration curve and found to be 1.504 MBq/mmol. Radiocounting was carried out to give >10^4 d.p.m. The error on the counts was then <2% at 95% confidence {*e.g.* for molar ratio 50 : 1 $[CE]_0 = 1 \times 10^{-2}$ M, $[Pr]_0 = 2.2 \times 10^{-4}$ M, Δ = 236 d.p.m., [CE.Pr] = 2.6×10^{-6} M}. [b] The molecular weight of maleyl cytochrome c was assumed to be the same as that of the unmodified protein.

Assuming the formation of a 1 : 1 complex‡ (n = 1, equation 1),† an average association constant was determined which, although not directly comparable with data obtained for simple organic molecules,[7] provides an order of magnitude for the association process. We also observed a trend of increasing K_s with decreasing temperature; however, for meaningful ΔH^0, ΔS^0, and ΔG^0 values more data points at several different temperatures are required. Measurements of K_s of

† The concentration of crown ether–protein complex [CE.Pr] was calculated from Δ in d.p.m., where d.p.m. is disintegrations per min,
Δ = d.p.m.$_{\text{bound crown}}$ − d.p.m.$_{\text{control}}$
d.p.m.$_{\text{bound crown}}$ = difference in counts between upper and lower compartments in presence of protein,
d.p.m.$_{\text{control}}$ = difference in counts between upper and lower compartments in absence of protein.

‡ The simplified case of n = 1 (equation 1) means one binding site per protein molecule has been considered in these studies. However, several binding sites are envisaged for the complexation of proteins, which would involve several data points in a Klotz or Scatchard plot (D. L. Hunston, *Anal. Biochem.*, 1975, **63**, 99).

cytochrome c with [1,2-$^{14}C_2$]-18-crown-6 reflect a small association in methanol (K_s 1—5 M^{-1}) compared with those of simple cationic species (K_s K^+–18-crown-6, 1.25×10^6 M^{-1}).[8]

In order to investigate the contribution of the alkylammonium ions of lysine residues to crown ether–protein binding, cytochrome c (containing 19 lysine groups) was modified by acylation with maleic anhydride[9] (pH_I unmodified, 9.83, pH_I modified, 4.2§). The maleyl cytochrome c, although soluble in methanol both in the presence and absence of crown ether, exhibited a lower K_s value compared with that of the unmodified protein (Table 2).

These preliminary studies concerning the selective solubilisation of proteins in non-polar solvents using crown ether type compounds are certain to offer a number of applications in biotechnology, in particular to the areas of protein separation, purification, and immobilisation.[10]

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§ The determination of the pH_I values of proteins was carried out according to LKB Ltd., technical note 1804 using iso-electric focusing on LKB AMPHOLINE ® PAGPLATES in the pH range 3.5—9.5.

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