Biochimica et Biophysica Acta, 480 (1977) 163—177

BBA 68023

CALF THYMUS ALKALINE PHOSPHATASE

II. INTERACTION WITH DETERGENTS

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(Received July 26th, 1976)

Summary

1. A number of detergents were used to dissolve calf thymus plasma membranes rich in alkaline phosphatase (orthophosporic-monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1) activity.

2. The Stokes' radius (r) of alkaline phosphatase in each detergent was measured by gel filtration. The size of the solubilized enzyme varied from r = 6.2 nm in sodium cholate to r = 8.3 nm in Berol EMU-043. With *N*-alkylsulphates, the apparent size increased with alkyl chain length, with r = 6.4 nm (C_9) and r = 7.3 nm (C_{12}). Tween 20 failed to solubilise the enzyme.

3. The effect of each detergent on the catalytic activity of alkaline phosphatase was determined. The non-ionic detergents Triton X-100, Nonidet P-40, Berol EMU-043, Tween 20 and the zwitterionic detergent Empigen BB increased V by 10—50% without substantially altering the K_m for *p*-nitrophenylphosphate. The bile salts sodium deoxycholate and sodium cholate decreased V and increased the apparent affinity of the enzyme for nitrophenylphosphate. Inhibition was concentration-dependent up to the critical micellar concentration, above which it remained constant (deoxycholate, 33%; cholate, 76%). Alkylsulphates (C_{8-12}) had no significant inhibitory effect during 24 h at 23°C.

4. Exchanging one detergent for another altered alkaline phosphatase activity to a state characteristic for the second detergent, e.g. the activity of cholate-inhibited alkaline phosphatase was restored to normal levels by excess of Triton X-100 and vice versa. The inhibitory effect of deoxycholate and cholate therefore result primarily from interactions between detergent and alkaline phosphate, rather than from selective removal of lipids from the enzyme.

5. Pure lecithin, lysolecithin and an ether-deoxylysolecithin each reactivated cholate-inhibited alkaline phosphatase in a concentration-dependent fashion. Cholesterol had no effect.

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6. The half-life ($t_{1/2}$) of membrane-bound alkaline phosphatase at 55° C was 64 min. With the exception of Berol, solubilisation in non-ionic detergents caused no marked change in this sensitivity. The enzyme became more labile in deoxycholate ($t_{1/2}$ = 31 min), but less labile in cholate ($t_{1/2}$ = 99 min). Alkylsulphates, which are strong denaturants, markedly increased the sensitivity of the enzyme to heat-inactivation (C_8, $t_{1/2}$ = 13 min; C_{9-12}, $t_{1/2} < 2$ min).

7. It is concluded that membrane-bound alkaline phosphatase is separated from most if not all of its neighbouring lipid moieties by these detergents, which bind to the solubilised enzyme. The number and character of molecules binding to the enzyme influence its size and shape, its susceptibility to inactivation and its catalytic activity.

Introduction

Detergents have played a key role in membrane investigations in recent years (review, ref. 1). The use of non-ionic and bile salt (so-called "mild") detergents has enabled cell membranes to be solubilised and many biologically-active membrane macromolecules to be isolated and partially characterised (e.g. refs. 2—5). "Strong" detergents, such as cetyltrimethylammonium chloride and particularly sodium dodecyl sulphate (SDS), bind co-operatively at room temperature to most proteins [6] and to virtually all proteins upon heating, resulting in denaturation and loss of biological activity [1,7,8]. In contrast, "mild" detergents such as Triton X-100 and sodium deoxycholate, which can solubilise membranes effectively, seem to be very inefficient in disrupting protein-protein interactions [1,5,9,10]. Many proteins, including some membrane-associated enzymes, retain their biological activity in the presence of high concentrations of Triton X-100 and deoxycholate [11—13]. Although the latter detergents bind to membrane proteins and to serum lipoproteins [3,4,12,14,15], they bind in insignificant amounts to nearly all soluble non-membrane proteins [3,8,15].

Despite extensive studies on the effects of detergents on protein structure [16,17] and their widespread use to solubilise membranes, particularly for the isolation of membrane-bound enzymes [3,11—13], there is little data available concerning the comparative effects of different detergents on the properties of a single membrane-bound enzyme. It is becoming increasingly evident, however, that the activity of numerous membrane-associated enzymes can be influenced by their immediate lipid environment within the membrane (review, ref. 18). For example, the activity of many membrane-bound enzymes can be significantly altered by treating membrane preparations with phospholipases, organic solvents or phospholipids [18—22], or the purified enzymes with synthetic lipids [23]. Several membrane-derived enzymes purified to apparent homogeneity are reported to contain small amounts of tightly-bound lipid [24,25].

The activity of membrane-associated enzymes can also be affected by detergents [11,18,26]. Most reports of this nature have however been limited to measurements of total activity, whereas changes in environmental conditions, such as loss of lipid or binding of detergent, may have pronounced effects on other properties, for example conformation, ligand requirements and substrate

affinity, as well as catalytic turnover. Considering the extensive use of detergents to solubilise membrane enzymes, it seems important to determine whether and if so, how, such properties of membrane-associated enzymes are affected by solubilisation in a given detergent.

This paper describes the effects of a number of different types of detergents on alkaline phosphatase (orthophosphoric-monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1), a membrane-associated enzyme present in calf thymocytes.

Materials and Methods

Chemicals

General. Sepharose 6B and Dextran Blue 2000 were purchased from Pharmacia (Uppsala, Sweden), tris(hydroxymethyl)aminomethane (Tris) was from Serva (Heidelberg), disodium paranitrophenylphosphate (*p*NPhP) from Boehringer (Mannheim) and diethanolamine from Merck (Darmstadt). $Na^{125}I$, carrier-free, 20—140 mCi/ml was obtained from Buchler Amersham (Braunschweig). All other reagents were of reagent or analytical grade.

Lipids. Cholesterol was obtained from Merck and arachidonoyl-Coenzyme A from General Biochemicals (Chagrin Falls, Ohio, U.S.A.). 1-Palmitoyl-*sn*-glycero-3-phosphorylcholine (lysophosphatidylcholine) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphorylcholine (phosphatidylcholine) were purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). An ether-deoxy analogue of lysophosphatidylcholine, 1-dodecyl-propanediol-3-phosphorylcholine (Et_{12}-H) was a generous gift from Dr. H. Weltzien of this institute. [^{14}C]lysolecithin [1-^{14}C]palmitoyl-*sn*-glycero-3-phosphorylcholine) was prepared enzymatically from [^{14}C]palmitic acid as previously described [27].

Detergents. Detergents were purchased from the following sources: Triton X-100 (scintillation grade), Tween 20 and sodium *n*-dodecyl sulphate (SDS), Serva (Heidelberg); Berol EMU-043 (an ethylene oxide adduct of fatty alcohols, mol. wt. approx. 700), MoDoKemi AB (Gothenburg, Sweden); Empigen BB (a mixture of 2-(*n*-alkyl-dimethylamino-)acetates, alkyl chain length C_{10-16}, mean mol. wt. 285), Marchon Ltd. (Cumberland, England); Sodium cholate, Fluka A.G. (Buchs, Switzerland); Sodium deoxycholate and the sodium salts of *n*-octyl-, *n*-nonyl- and *n*-decyl-sulphate, Merck (Darmstadt). Nonidet P-40 was a gift from the Deutsche Shell Chemie Gesellschaft MBH (Munich). All concentrations of detergents expressed as % refer to w/v. Approximate critical micellar concentrations are: Triton X-100, 0.015%; Tween 20, 0.006%; deoxycholate, 0.2%; cholate, 0.6%; octylsulphate, 2.3%; nonylsulphate, 1.5%; decyl sulphate, 0.8% and dodecyl sulphate, 0.2% [1,28]. These values are only approximate as critical micelle concentration can be affected by temperature, ionic strength and pH [1,17].

Marker proteins. Bovine serum albumin, horse splenic ferritin, bovine fetuin and horse heart myoglobin were purchased from Sigma Chemical Co.. Rat IgM and rabbit IgG were purified from the respective sera by precipitation in 45% saturated ammonium sulphate, followed by chromatography on Sephadex G-200 and DEAE-cellulose [29]. Stokes' radii (r) were calculated from published data [30] according to: $r = (f/f_o) \cdot (3\nu \cdot M_r/4\pi \cdot N)^{1/3}$, where f/f_o = frictional coef-

ficient, v = partial specific volume, M_r = molecular weight and N is Avogadro's number [31].

Iodination of marker proteins

Small amounts (10—20 μg) of protein were trace-labelled with ^{125}I using chloramine-T as described elsewhere [32].

Preparation of thymocyte upper microsomal band membranes

The details for preparing thymocyte suspensions, cell disruption and subcellular fractionation have been described elsewhere (ref. 33 and Ey and Ferber in preparation). Briefly, cell suspensions prepared from thymi of freshly slaughtered calves were treated by the "nitrogen cavitation" method [33,34] and the microsomes isolated by differential centrifugation of the "homogenate". The microsomal material was then further fractionated according to particle density by centrifugation in discontinuous sucrose density gradients (45%/35%, w/v/buffer; Beckman Ti60 rotor, 50 000 rev./min, 5° C, 2 h). The membraneous material banding between the buffer and 35% sucrose layer (d = 1.132) was washed with and resuspended in 10 mM NaCl/25 mM KCl/10 mM Tris · HCl (pH 8.2) containing 15 mM NaN_3 (Buffer A). This material (the upper microsomal band) contained 1.01 ± 0.11 μmol of phospholipid and 0.73 ± 0.12 μmol of cholesterol (mean ± S.D., approx. 0.62 mg total lipid) per mg of protein and was stored unfrozen at 0° C. No changes in total alkaline phosphatase activity or specificity could be detected under these conditions, even after several months. No evidence was found to suggest that alkaline phosphatase was in any way degraded or altered during storage. However, a new preparation was made each week and fresh preparations were used in most experiments.

Incorporation of [^{14}C]lysophosphatidylcholine into upper microsomal band membranes

Upper microsomal band membranes were labelled by incorporation of [^{14}C]-lysolecithin (52 nmol) for 10 min at 37° C in 1.0 ml of 0.1 M phosphate buffer (pH 7.0) in the presence of arachidonoyl-Coenzyme A (30 nmol). The suspension was then diluted with and washed at 4° C by centrifugation and resuspension (twice) in Buffer A. Thin-layer chromatography of a lipid extract [27] of these labelled membranes on 0.75-mm silica plates (20 × 16 cm, Kieselgel H., Merck, Darmstadt) using chloroform/methanol/water (65 : 40 : 10, v/v) as the developing solvent indicated that more than 91% of the membrane-associated radioactivity was present as phosphatidylcholine.

Protein determination

Protein was determined by the ninhydrin method [35].

Assay of alkaline phosphatase

Except where indicated otherwise, alkaline phosphatase was assayed in a final volume of 1.0 ml containing, in addition to enzyme material, 1 mM $MgCl_2$, 0.5% Triton X-100, 0.1 M diethanolamine · HCl (pH 9.5) and 2.5 mM *p*NPhP. Routine duplicate assays were usually incubated for 15—30 min at 37° C before being terminated by addition of 0.2 ml of 5 M NaOH. Activity was calculated

from the absorbance at 405 nm, using an extinction coefficient for nitrophenol of 18 200 $l \cdot mol^{-1} \cdot cm^{-1}$. The activity at 37° C was constant for at least 90 min provided that less than 5% of the substrate was hydrolyzed.

Results

Size of detergent-solubilized alkaline phosphatase

Detergents differ markedly in their capacity to solubilise various types of lipid [1,36–38] and in their ability to bind to proteins [3,15]. It was therefore important to determine first how effectively membrane lipids, as well as alkaline phosphatase, were solubilised by each detergent and secondly, the actual size of the detergent-solubilised alkaline phosphatase molecules.

Fig. 1 depicts the elution profiles obtained by chromatography of [^{14}C]phos-

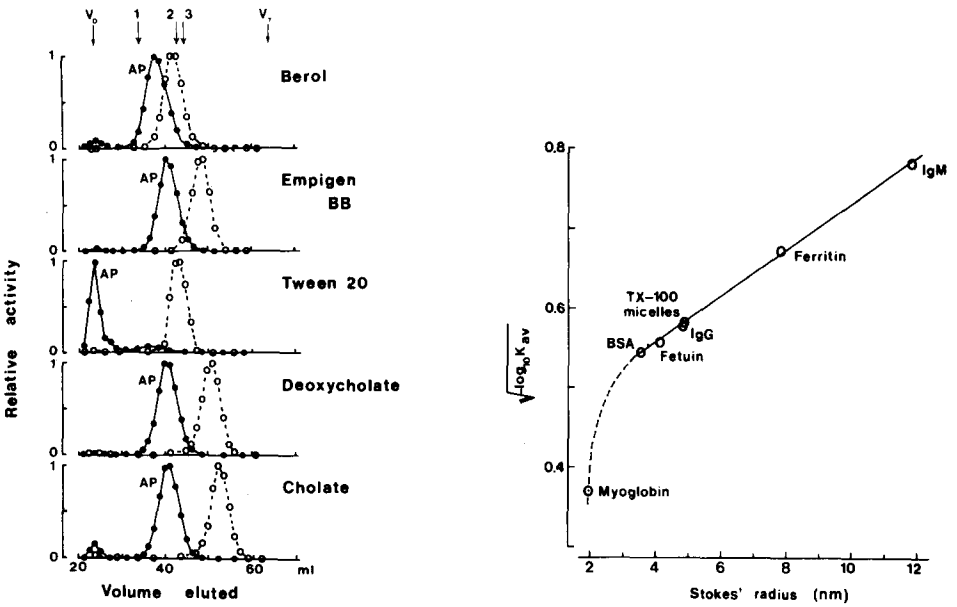

Fig. 1. Gel filtration on Sepharose 6B of microsomal membranes solubilised in detergent. Samples of [^{14}C]phosphatidylcholine-labelled upper microsomal band membrane (1.0 ml containing 0.17 mg protein, 0.105 mg lipid and 30 mg of detergent) in eluant buffer (50 mM NaCl/15 mM NaN_3/10 mM Tris · HCl, pH 8.2) * were applied to the top of a 1.07 × 65-cm column of Sepharose 6B equilibrated and eluted with buffer containing the same detergent at 1% concentration. The column was eluted at 23° C at a flow rate of 5.32 ml/h. Samples also contained as intrinsic markers, Dextran Blue 2000 (0.5 mg/ml), ^{125}I-labelled IgM, IgG and bovine serum albumin (approx. 5000 cpm each) and *p*-nitrophenol (0.05 mM). Aliquots of each 1.24-ml fraction were assayed for alkaline phosphatase (AP) activity (●———●), ^{14}C (○- - - - -○), ^{125}I and absorbance at 620 nm and 405 nm. The elution positions for Dextran Blue (V_0), IgM (1), IgG (2), bovine serum albumin (3) and nitrophenol (V_T) are indicated (arrows).

Fig. 2. Relationship between Stokes' radius and elution position of marker substances on Sepharose 6B. Each marker was applied separately to the column described in Fig. 1 and eluted in the absence of detergent. Stokes radii (nm) used to construct the graph were: myoglobin (1.9), bovine serum albumin (BSA) (3.5), fetuin (4.1), IgG (4.9), ferritin (7.8) and IgM (11.8). To determine the mean elution position of Triton X-100 (TX-100) micelles, samples containing 0.5% Triton X-100 were applied to the column after it had been equilibrated with 0.05% Triton X-100. The mean Stokes' radius of these micelles is 4.85 nm [40]. $K_{av} = (V_e - V_0)/(V_T - V_0)$ where V_e = elution volume of solute, V_0 = void volume and V_T = total volume of gel bed [31,39].

* With ionic detergents, 0.1 M NaCl was used to minimise ionic interactions.

phatidylcholine-labelled membranes through Sepharose 6B in the presence of one of several detergents. The profile for Triton X-100 (not shown) has been presented elsewhere (Ey & Ferber, Biochim. Biophys. Acta, in the press). In each case, the membranes were solubilised with a large excess of detergent (w/w ratio, detergent: membrane lipid = approx. 280) at high concentration (3%) and chromatographed in the presence of the same detergent at a concentration (1%) which, except for octyl and nonyl sulphate, was greater than the critical micelle concentration [1,28]. With the exception of Tween 20, each of the detergents tested completely solubilised alkaline phosphatase and membrane phospholipids, so that upon chromatography a single peak was obtained for each component in which essentially all of the activity applied to the column was recovered. Only trace amounts of alkaline phosphatase and virtually no labelled phospholipid was excluded by the gel, whereas when membrane samples not treated with detergent were chromatographed in the absence of detergent, all alkaline phosphatase activity and all phospholipid radioactivity eluted with the void volume. It is noteworthy that Tween 20 completely solubilised the membrane phospholipids although it failed to solubilise alkaline phosphatase to a nonaggregated form. The latter was excluded by Sepharose 6B, indicating that its aggregate weight was $>4 \cdot 10^6$ daltons (Pharmacia, product information).

The elution position of the labelled phospholipid peak was characteristic of the solubilising detergent. This is consistent with the concept that detergent-lipid mixed micelles are formed, their size being determined by the nature of the detergent as well as the type of lipid and the detergent/lipid ratio [1,17]. The elution position of alkaline phosphatase was also dependent on the solubilising detergent. This could be due to incomplete removal of lipids from alkaline phosphatase by some detergents and/or binding of detergent to the enzyme molecules. The actual dimensions of the solubilised enzyme molecules were ascertained by calculating their Stokes' radii (r) from a plot of r vs. $(-\log_{10} K_{av})^{1/2}$ (Fig. 2) [31,39].

The relationship obtained using marker substances of known dimensions is depicted in Fig. 2 and the calculated Stokes' radius for alkaline phosphatase in each detergent is given in Table I. The size of the solubilised enzyme was minimal in sodium cholate (r = 6.2 nm) and maximal in Berol EMU-043 (r = 8.3 nm).

Effect of solubilisation on the catalytic properties of alkaline phosphatase

It has been shown (Ey & Ferber, Biochim. Biophys. Acta, in the press) that calf thymus alkaline phosphatase is a relatively non-specific orthophosphomonoesterase with a high affinity for *p*NPhP. The effect of each detergent on K_m and V using *p*NPhP as substrate was determined by measuring the rate of hydrolysis at different substrate concentrations in the presence of detergent (1%). The results are shown in Table II.

All the non-ionic detergents as well as the zwitterionic detergent Empigen BB increased V by 7—54%. Whether this apparent stimulation is real or due to release of latent enzyme as a result of membrane disruption has not been investigated. The latter detergents had little effect of K_m for *p*NPhP, the greatest effect being a 78% increase in apparent affinity with Berol EMU-043.

In contrast to the non-ionic detergents, sodium cholate and deoxycholate

TABLE I

MOLECULAR SIZE OF ALKALINE PHOSPHATASE AFTER SOLUBILIZATION OF MICROSOMAL MEMBRANES IN DETERGENT

Values represent Stokes' radii for alkaline phosphatase calculated from chromatography through Sepharose 6B at pH 8.2 in the presence of 1% of the indicated detergent (for details, see Fig. 1).

Detergent (1%, w/v)	Stokes' radius (nm)
None	— *
Triton X-100	7.7
Berol EMU-043	8.3
Tween 20	— *
Empigen BB	6.4
Deoxycholate	6.4
Cholate	6.2
Octyl sulphate	— **
Nonyl sulphate	6.4
Decyl sulphate	6.9
Dodecyl sulphate (SDS)	7.3

* Totally excluded by Sepharose 6B
** Incompletely solubilized.

had a pronounced influence on the catalytic constants. Both detergents decreased V (deoxycholate, by 33% and cholate, by 76%) and increased the apparent affinity for *p*NPhP, (deoxycholate, 4-fold; cholate, 10 fold). The decreased rate of substrate hydrolysis induced by these bile salt detergents cannot be due to permeability or latency effects, but must be a consequence of removal from the enzyme of lipids or other factors and/or binding of detergent to the enzyme. Octyl and nonyl sulphate at room temperature caused a slight decrease V and an increase of K_m. The effect of SDS is not shown in Table II, but this detergent had no inhibitory effect on hydrolytic rate at room temperature.

TABLE II

EFFECT OF DETERGENTS ON THE KINETIC CONSTANTS OF ALKALINE PHOSPHATASE

Fixed amounts of upper microsomal fraction membrane (16—34 μg protein/ml) were incubated for 24 h at 25°C in 50 mM NaCl/10 mM Tris · HCl, pH 8.2 containing 15 mM NaN_3 and detergent as indicated. Enzyme activity was then assayed at 37°C at 8 different substrate (*p*NPhP) concentrations (0.1—1.0 mM) in 50 mM diethanolamine · HCl (pH 9.5) containing 1 mM $MgCl_2$ and the respective detergent at a final concentration of 1%. K_m and V were calculated by double reciprocal plots [41].

Detergent (1%, w/v)	K_m (μM)	Relative V (%)
None	41	100
Triton X-100	30	154.4
Nonidet P-40	33	117.6
Berol EMU-043	23	106.8
Tween 20	30	154.4
Empigen BB	39	136.7
Deoxycholate	10	67.2
Cholate	4	24.4
Octyl sulphate	84	96.6
Nonyl sulphate	50	81.3

Effect of detergent exchange on rate of substrate hydrolysis

Because the rate of substrate hydrolysis could be increased or decreased depending on which detergent was used to solubilise membrane-bound alkaline phosphatase, it was decided to investigate the reversibility of these effects by exchanging one detergent for another. Thus, membrane preparations were dissolved in buffer containing either no detergent or an excess (w/w ratio, detergent : membrane lipid = 160) of Triton X-100, deoxycholate, cholate or SDS. An aliquot of each of these solutions was then diluted into buffer containing no detergent, the same detergent, or a different detergent at the same w/v concentration. After 2 h at room temperature, residual alkaline phosphatase activity was measured. The results of this experiment are presented in Table III.

When the membrane preparation was dissolved in detergent and diluted into the same detergent, the effect was as expected from Table II. However, when the preparation was first treated with Triton X-100 or SDS (both of which apparently stimulate alkaline phosphatase activity) or with deoxycholate (which causes partial inhibition) and then diluted into an excess of cholate, the enzyme activity was reduced to the level obtained by treatment with cholate only. As dilution into excess cholate is most unlikely to have replaced (on to the enzyme) any lipids removed by Triton X-100, SDS or deoxycholate, the inhibition caused by cholate must be due either to removal from alkaline phosphatase of essential lipids which are not removed by the other three detergents or to the effects of cholate itself binding to the enzyme. A similar conclusion can be drawn for deoxycholate, although the inhibition is less severe. However, when membranes treated with either deoxycholate or cholate were diluted into excess Triton X-100 or SDS, complete reactivation of hydrolytic activity was achieved. Such reactivation by Triton X-100 or SDS cannot be due to the replacement on alkaline phosphatase of lipids removed by cholate or deoxycholate and one can only conclude that the reactivating detergents are removing cholate or deoxycholate molecules from the enzyme. Thus it appears

TABLE III

EFFECT OF DETERGENT EXCHANGE ON ALKALINE PHOSPHATASE ACTIVITY

To determine the effect on alkaline phosphatase activity of replacing the detergent used to solubilize the enzyme by a second detergent, upper microsomal fraction membranes were incubated at 0.1 mg of protein/ml in buffer (50 mM NaCl/15 mM NaN_3/10 mM Tris · HCl, pH 8.2) containing detergent A as indicated. After 20 h at 23°C, 0.04 ml of each of these solutions was mixed with 0.5 ml of buffer containing 1% of detergent B. After incubation at 23°C for 2 h, 0.5 ml of 2 mM $MgCl_2$/0.5 M diethanolamine · HCl (pH 9.5) and 0.1 ml of 25 mM *p*NPhP were added and alkaline phosphatase activity determined at 37°C.

% Residual activity

Detergent A (1%, w/v)	Detergent B				
	None	Triton X-100	Deoxycholate	Cholate	SDS
None	100	—	—	—	—
Triton X-100	131	131	56	24	140
Deoxycholate	97	121	53	24	138
Cholate	63	76	48	19	119
SDS	136	132	54	25	132

that the bile salt detergents bind to alkaline phosphatase and cause partial inhibition of enzymatic activity. This inhibition is relased if the bound detergent molecules are removed from the enzyme, either by dilution into detergent-free buffer (Table III) or by a second, non-inhibitory detergent which may replace the bile salt on the enzyme molecule.

Effect of detergents on the susceptibility of alkaline phosphatase to thermal inactivation

Denaturation of proteins involves the disruption of non-covalent, structure-stabilising bonds which leads to loss of secondary and higher structure and of biological activity [6]. Changes in the non-covalent bond structure of an enzyme can therefore often be detected by measuring its susceptibility to heat denaturation. To determine whether solubilisation of membrane-bound alkaline phosphatase by detergent caused any alteration in the susceptibility of the enzyme to heat, samples of membrane in buffer containing detergent at 1% concentration were incubated at a number of temperatures and the rate of inactivation of alkaline phosphatase measured (Table IV).

In the absence of detergent, (membrane-bound) alkaline phosphatase was stable at 45°C, but became slowly inactivated at 50°C and rapidly at 60°C ($t_{1/2}$ = 407 min and 3.9 min, respectively). In the non-ionic detergent Berol EMU-043, the half-life of the enzyme over the temperature range 55—62.5°C was approximately 40—50% of that in the absence of detergent. The other non-ionic detergents, as well as Empigen BB, had a less marked influence, reducing $t_{1/2}$ by less than 30%. The two bile salt detergents unexpectedly produced

TABLE IV

EFFECT OF DETERGENTS ON THE SUSCEPTIBILITY OF ALKALINE PHOSPHATASE TO THERMAL INACTIVATION

Samples of upper microsomal fraction membrane (0.1 ml; 15—34 μg protein/ml) were incubated at each of the indicated temperatures in 50 mM NaCl/10 mM Tris · HCl (pH 8.2) containing 15 mM NaN_3 and detergent as indicated. At various times, tubes were removed and placed in ice. To measure residual enzyme activity, each tube received 0.8 ml of assay buffer and after 5 min preincubation at 37°C, 0.1 ml of 25 mM *p*NPhP. The reaction was stopped after 60 min at 37°C by the addition of 0.2 ml 5 M NaOH. A straight line was obtained for each series of results by plotting $\log_{10}(N_0/N_t)$ versus time, where N_0 = initial activity and N_t = activity at time t. Values represent the mean half-life (in minutes) determined from 2 separate experiments.

Detergent (1%, w/v)	Temperature (°C)					
	45.0	50.0	55.0	57.5	60.0	62.5
None	∞	407	64.1	13.0	3.90	1.61
Triton X-100	∞	205	57.3	13.0	2.95	1.43
Nonidet P-40	—	—	61.1	13.0	2.71	1.72
Berol EMU-043	—	—	26.7	4.0	2.11	1.07
Tween 20	—	—	67.6	14.9	3.59	1.72
Empigen BB	—	—	49.7	13.0	4.91	1.59
Deoxycholate	∞	224	30.9	10.0	3.62	0.94
Cholate	∞	637	98.6	24.8	9.29	2.06
Octyl sulphate	—	—	12.7	4.4	1.10	0.54
Nonyl sulphate	—	—	<2	<0.8	≤0.29	<0.1
Decyl sulphate	—	—	<2	<0.8	<0.1	<0.1
Dodecyl sulphate	—	—	<2	<0.8	<0.1	<0.1

opposite effects. Over the 55—62.5° C range, the mean half-life of the enzyme in deoxycholate was approximately 66%, whereas in cholate it was 173% of the control value (no detergent). Thus, deoxycholate increased and cholate decreased the susceptibility of alkaline phosphatase to heat inactivation.

The effect of the alkyl sulphates was the most marked of all the detergents tested. The mean half-life (55—62.5° C) relative to that in the absence of detergent was 29% in octyl sulphate, but less than 3% in nonyl, decyl and dodecyl sulphates. This is consistent with the strong denaturing properties of these surfactants [1,6—8].

Reactivation by addition of synthetic lipids

Solubilisation in certain kinds of detergents removes most if not all lipids from membrane proteins [36,37]. It has been found, however, that equilibration of added lipids between lipophilic sites on molecules of purified adenosine triphosphatase and free lipid is facilitated by low concentrations of sodium cholate [23]. Since the results presented above indicated that cholate binds to alkaline phosphatase molecules, possibly in substitution for membrane lipids removed during solubilisation of the enzyme, the influence of purified lipids on the activity of the cholate-inhibited enzyme was investigated.

Thus, membrane samples were solubilised with a 550-fold w/w excess of cholate : membrane lipid in order to deplete the cholate-solubilised alkaline phosphatase molecules of membrane lipids. Aliquots of this solution were then mixed with different amounts of synthetic lipid such that the larger amounts were in molar excess of cholate. Alkaline phosphatase activity was then determined and the results are shown in Fig. 3.

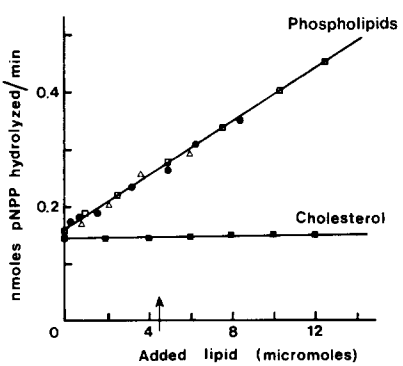

Fig. 3. The activity of cholate-inhibited alkaline phosphatase upon exposure to purified lipids. Upper microsomal fraction membranes were dissolved in 0.1 M NaCl/15 mM NaN_3/10 mM Tris · HCl (pH 8.2) containing 1.0% sodium cholate and incubated 6 h at 20° C. Aliquots of 0.2 ml (containing 5.6 μg protein, 3.5 μg membrane lipid and 2.0 mg (4.6 μmol) cholate) were added to small glass tubes in which different amounts of purified lipid dissolved in chloroform/methanol (2 : 1, v/v) had been air-dried and resuspended in water. Each tube was sealed with parafilm, vigorously shaken and incubated for 10 h at 37° C. Alkaline phosphatase activity was then assayed at 37° C by adding 0.5 ml of 2 mM $MgCl_2$/0.5 M diethanolamine · HCl (pH 9.5) and 0.1 ml of 25 mM *p*-nitrophenylphosphate (pNpp). Reaction was stopped after 30 min by adding 0.2 ml of 5 M NaOH and 1.0 ml of 10% SDS, the latter to facilitate clarification of samples containing large amounts of lipid. Lipids tested were: Cholesterol (■), Et_{12}-H (□), lysophosphatidylcholine (●), phosphatidylcholine (△). The point of molar equivalence between cholate and added lipid is indicated (arrow).

The activity of the cholate-inhibited enzyme increased upon exposure to lysophosphatidylcholine, the increase being linearly porportional to the amount of lipid added. An ether-deoxylysophosphatidylcholine analogue, Et_{12}-H, produced an identical dose-dependent reactivation. Because both of these compounds are very water-soluble and possess detergent properties [17,42], the effect of lecithin, which is almost water-insoluble, was tried. As can be seen, an identical effect was produced. These results were in marked contrast to the inability of cholesterol to activate the enzyme.

Discussion

It has been estimated (ref. 1, pp. 59, 60) that for Triton X-100, SDS and deoxycholate, a detergent : lipid (w/w) ratio of approximately 2 : 1 is necessary to accomplish a breakdown in membrane lamellar structure. Although this may be achieved with some detergents at concentrations less than the critical micelle concentration, complete solubilisation of membrane lipids into mixed micelles probably requires detergent concentrations greater than the critical micelle concentration as well as a high detergent : lipid ratio [1,43]. Maximum protein solubilisation of rat liver membranes by deoxycholate is achieved at a deoxycholate : phospholipid ratio (R) of 2, but complete phospholipid solubilisation requires R = 14—16 [36]. For $R < 2$, sterols are solubilised in slightly greater amounts than phospholipids and the whole membrane cholesterol goes into solution at R = 14—16. However, solubilisation of esterified cholesterol is never complete [36].

In the present work, detergent : lipid ratios of 160 to 550 have been used, in most cases with micellar concentrations of detergent, so that solubilisation of membrane lipids will have been maximal. It is possible, even at these high detergent levels, that solubilised membrane proteins are not entirely lipid-free. However, serum lipoprotein as well as erythrocyte and virus membrane proteins can be obtained essentially lipid-free by treatment with micellar concentrations of Triton X-100 or deoxycholate [15] and there is no reason why this situation should not apply in the present work. If this is the case, then the difference in Stokes' radii of alkaline phosphatase in the various detergents must be due to differences in the amount of detergent bound to the enzyme. This would be consistent with the findings that Triton X-100, deoxycholate and cholate bind to lipid-depleted proteins in significant amounts, probably to lipophilic sites normally occupied by lipid [1,3,4,8,12].

If the differences in Stokes' radius of alkaline phosphatase in each detergent are due only to differences in the amount of bound detergent, a rough estimate of this quantity can be made. Purified alkaline phosphatases from various sources as well as the Triton X-100-solubilised calf thymus enzyme (Ey and Ferber, Biochim. Biophys. Acta, in the press) have been found to have an $s_{20,w}$ of between 6 and 7 [44,45]. If it is assumed that the partial specific volume (v) of the cholate- and deoxycholate-alkaline phosphatase complexes is about 0.73 [4] and that the sedimentation coefficient of each of these complexes is about 6.5 S, it can be calculated from their Stokes' radii (Table I) that their respective molecular weights would be 169 000 and 174 000 (Table V). This is compatible with the molecular weight range of purified alkaline

TABLE V

ESTIMATION OF BOUND DETERGENT PER MOLE OF ALKALINE PHOSPHATASE

Detergent	Detergent-enzyme complex			Bound detergent/mol alkaline phosphatase [c]	
	Stokes' radius (nm) [a]	Assumed $\bar{\nu}$	Calculated mol. w.t. [b]	Daltons	mol
Cholate	6.2	0.73 [e] (0.78)	169 000 (207 000)	9 000 (47 000)	21 (109)
Deoxycholate	6.4	0.73 [e]	174 000	14 000	34
Triton X-100	7.7	0.78 [f]	257 000	97 000 [d]	152
Berol EMU-043	8.3	0.78	277 000	117 000	167
Empigen BB	6.4	0.75	188 000	28 000	98
Nonyl sulphate	6.4	0.75	188 000	28 000	114
Decyl sulphate	6.9	0.75	203 000	43 000	165
Dodecyl sulphate	7.3	0.75	215 000	55 000	191

[a] From Table I.

[b] Assuming $s_{20,w}$ = 6.5 [44,45]. Mol. wt. = $6\pi\eta_{20,w} \cdot N \cdot r \cdot s_{20,w}/(1 - \nu \cdot \rho_{20,w})$ where $\eta_{20,w}$ is viscosity of water at 20°C, N is Avogadro's number, r is Stokes' radius, $s_{20,w}$ is sedimentation coefficient in water at 20°C, ν is partial specific volume, $\rho_{20,w}$ is density of water at 20°C.

[c] Assuming mol. wt. (enzyme) = 160,000 [44—46].

[d] Mean Triton X-100 micellar weight = 291 000, equivalent to 457 molecules (calculated from data of ref. 40).

[e] Ref. 4.

[f] Ref. 3,4.

phosphatases from several sources (150 000—170 000; refs. 44—46). For a mean enzyme mol. wt. of 160 000, there would be of the order of 20—100 mol of cholate or deoxycholate bound per mole of enzyme. It is clear from the results of Table III that some cholate and deoxycholate must bind to alkaline phosphatase.

The estimates of Table V also indicate that large quantities of the non-ionic detergents Triton X-100 and Berol bind to alkaline phosphatase. The estimated binding (approx. 0.6% w/w, detergent : protein) is similar to levels of binding of Triton X-100 to other membrane proteins [3,4,12,15]. Tanford [17,43] has proposed that solubilised membrane proteins might in some circumstances be incorporated within detergent micelles, rather than merely saturated with detergent monomers. This seem unlikely with alkaline phosphatase and Triton X-100 (mean micellar weight, 291 000: Table V), as the mol. wt. of the alkaline phosphatase-Triton X-100 complex would be 160 000 + 291 000 = 451 000, which is considerably larger than calculated (Table V). However, the large quantity of Triton X-100 bound to the solubilised enzyme may be associated in a micelle-like structure and much of the detergent may not be in contact with the protein.

The number of bound alkyl sulphate molecules increased with alkyl chain length, suggesting that a corresponding increase in co-operative interaction occurs [8]. However, the enzyme retained its full catalytic activity in these strong detergents at room temperature (Tables II and III), as has been shown previously for rat liver alkaline phosphatase in SDS [11]. Tween 20 failed to

fully solubilise alkaline phosphatase, although it completely solubilised labelled membrane phospholipid. This is consistent with the reported selective solubilising capacity of this detergent [1,38] and the fact that it has a hydrophilic/lipophilic balance number outside the range optimal for membrane solubilisation [37].

Most of the detergents tested had only minor effects on alkaline phosphatase catalytic activity. The apparent stimulation of hydrolytic rate by Triton X-100 and SDS has also been reported for the rat liver enzyme [11]. In comparison to the other detergents, the effects of cholate and deoxycholate were most marked (Table II). As has already been concluded from the results (Table III), these effects can only be due to the binding of these detergents to the lipid-depleted enzyme, which may affect the active centre directly or allosterically. It is questionable whether these detergents are acting as analogues of cholesterol, but if so, it could indicate that the normally-uninhibited membrane-bound enzyme does not associate with membrane cholesterol.

The probability that large quantities of Triton X-100 and Berol bind to alkaline phosphatase (above), the known ability of different detergents to replace one another on lipophilic sites on proteins [4,15] and the fact that Triton X-100 and SDS can reactivate cholate- or deoxycholate-inhibited alkaline phosphatase strongly suggests that these detergents compete for common binding sites on the enzyme molecule. If the estimated amount of each detergent bound to alkaline phosphatase is correct, then many more molecules of Triton X-100, Berol and SDS bind per mole of enzyme than do those of cholate or deoxycholate. These additional molecules may bind to other sites on the enzyme or possibly to pre-bound detergent molecules by micelle-like detergent-detergent rather than protein-detergent interactions [1,17].

Most of the non-ionic detergents had little influence on the thermal inactivation of alkaline phosphatase. Triton X-100, which caused a slight decrease in $t_{1/2}$, has been found to stabilize viral DNA polymerase against inactivation by heat [26]. The effect of the alkyl sulphates reflects their strong denaturing properties, particularly upon heating [1,17]. However, the contrasting effects of cholate and deoxycholate were puzzling (Table IV). It is possible that cholate stabilizes the enzyme in a more restricted conformation which interferes with catalytic turnover but is more resistant against heat denaturation. Deoxycholate, on the other hand, also reduces catalytic turnover, but decreases thermal stability. More information regarding the number of common and unique binding sites for these two detergents needs to be obtained to clarify this phenomenon.

The reactivation of cholate-inhibited alkaline phosphatase by phospholipids was in marked contrast to the lack of effect by cholesterol. Although the extremely low solubility of cholesterol in water must be borne in mind, the small amount of cholate present in the mixture, particularly at low concentrations of cholesterol, should have enabled some equilibration with lipophilic enzyme sites to occur. This seems to have been achieved with phosphatidylcholine. The results suggest that membrane-bound alkaline phosphatase, which is normally uninhibited, may be closely associated with phospholipids rather than cholesterol. Other membrane enzymes are known to be activated by phospholipids of various kinds [18].

The results of this study indicate that detergents are capable of altering various properties of membrane-bound enzymes. These effects are related to structural or conformational changes brought about by the removal of enzyme-bound lipids and/or the binding of detergent molecules to the lipid-depleted enzyme. The observed effect of a given detergent depends on its ability to exchange places with enzyme-bound lipids and on its chemical structure.

Acknowledgements

We wish to thank Miss Käthe Hansen for her excellent skilled technical assistance and Professor H. Fischer for providing the opportunity for P.E. to undertake this work at the M.P.I. (Freiburg). This investigation was supported by the Deutsche Forschungsgemeinschaft and the Max-Planck-Gesellschaft zur Forderung der Wissenschaften (E.F.) and by the Alexander von Humboldt-Stiftung (P.E.).

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