DETERGENTS MODIFY THE FORM OF ARRHENIUS PLOTS OF 5'-NUCLEOTIDASE ACTIVITY

I. DIPPLE, K. R. F. ELLIOTT* and M. D. HOUSLAY

*Department of Biochemistry, University of Manchester Institute of Science and Technology, PO Box 88, Manchester, M6O 1QD and *Department of Biochemistry, University of Manchester, Oxford Road, Manchester, M13 9PL, England*

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1. Introduction

5'-Nucleotidase is an intrinsic glycoprotein of rat hepatocyte plasma membranes [1–3] with its active site on the external surface of the membrane [1,4–6]. The enzyme has been solubilised with non-ionic detergents, and purified to homogeneity as a lipoprotein complex with tightly-associated endogeneous sphingomyelin [3,7]. Solubilized preparations have been reported to have mol. wt 52 000–237 000 [3,8–12] which may be a reflection of the formation of aggregate species.

The activity of the enzyme is modulated by the physical properties of the bilayer in that Arrhenius plots of its activity show a well defined break at 28°C, characteristic of a lipid phase separation occurring in the outer half of the bilayer [13–15]. Addition of the local anaesthetic, benzyl alcohol, increases the fluidity of the bilayer, and depresses the temperature of the lipid phase separation by about 6°C, achieving activation of 5'-nucleotidase and a shift in the temperature of the break in its Arrhenius plot down to about 22°C [16].

Many membrane-bound enzymes are solubilized using detergents, although little is known about the interaction of detergents with the protein species or any effect on the activity and properties of the enzyme. In this paper we demonstrate that soluble preparations of 5'-nucleotidase obtained using different detergents exhibit markedly different Arrhenius plots, the form of which appears to be related to the physical properties of the detergent.

2. Materials and methods

Rat liver plasma membranes were used as a source of 5'-nucleotidase. The membranes were prepared from male (200–300 g) Sprague Dawley rats [17]. Membranes were stored in liquid nitrogen at protein conc. 6–20 mg/ml in 1 mM $KHCO_3$, pH 7.2.

Soluble enzymes were prepared using the following detergents; Lubrols 12A9, 17A10, N13 and G, Triton X-100 and Na-deoxycholate. Lubrol preparations were rendered soluble with 2.5% detergent as in [18]. A protein to detergent ratio of 1:4 was maintained for all Lubrol preparations. Na-deoxycholate preparations were made using 0.5% detergent, and Triton X-100 preparations using 2.5% detergent. The upper clear fraction obtained after centrifugation for 1 h at 100 000 × *g* (4°C) was taken as the source of enzyme activity (in the case of Lubrol G this fraction was opaque). These fractions were either assayed immediately or could be stored for several weeks in liquid nitrogen without loss of activity. 5'-Nucleotidase was assayed radiometrically using [^{3}H]adenosine 5'-monophosphate as substrate [19] or using a coupled spectrophotometric assay [10]. In all cases initial rate determinations were made and the pH was kept constant at pH 7.4. K_m values were determined at 30°C.

Molecular weights were estimated by gel filtration using Sephadex G-200 equilibrated with 150 mM KCl, 1 mM EDTA and 50 mM Tris–HCl at final pH 7.6 (buffer A). Lactate dehydrogenase, pyruvate kinase, alcohol dehydrogenase, glutamate dehydrogenase, glycerol 3-phosphate dehydrogenase,

malate dehydrogenase, haemoglobin and cytochrome *c* were used as standards to calibrate the column.

Protein concentrations were determined as in [20]. This method was unaffected by the presence of detergents at the concentrations used.

Lubrols 12A9, 17A10, N13 and G were kind gifts from ICI. [^{3}H]Adenosine 5′-monophosphate was obtained from Amersham. Triton X-100 was obtained from Koch-Light. All other reagents were obtained from Sigma Chemical Co., London and BDH, Poole and were of analytical reagent grade where available.

3. Results

Detergent preparations were assumed soluble on the basis of the following criteria. Enzyme activity was not sedimented by centrifugation for 1 h at 100 000 × *g* and 5′-nucleotidase activity was found in the included volume of Sephadex G-200.

Molecular weights of ~ 250 000 were obtained by Sephadex G-200 gel filtration with a minor fraction of mol. wt ~ 65 000. A minor peak of activity (<15%) was found in the void volume. This peak was not observed when using a column re-equilibrated with 0.5% detergent in buffer A. It was thought that the void volume peak was due to the formation of aggregated enzyme spieces produced during gel filtration.

Recoveries of activity of greater than 100% were obtained for the enzyme solubilised using Na-deoxycholate and the various Lubrols (with the exception of Lubrol N13). However Triton X-100 and Lubrol N13 produced recoveries of significantly less than 100% activity (table 1).

K_m values for the native and the various soluble enzyme preparations showed no significant differences having values of 17–25 μM.

The Arrhenius plot of the native enzyme was clearly biphasic (fig.1), showing a break at 27.5°C. Treatment with Na-deoxycholate (fig.1) and Lubrol G, (fig.2) resulted in soluble enzymes which exhibited linear Arrhenius plots of 5′-nucleotidase activity. However,

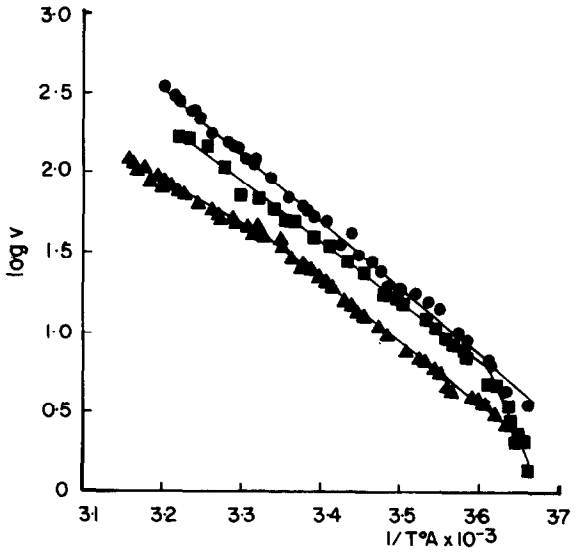

Fig.1. Arrhenius plots of native, deoxycholate-solubilized and Triton X-100-solubilized 5′-nucleotidase activity. Activity is measured as nmol/min/mg protein. Activation energies are given in table 1. Native enzyme (▲). Triton X-100 (■). Deoxycholate (●).

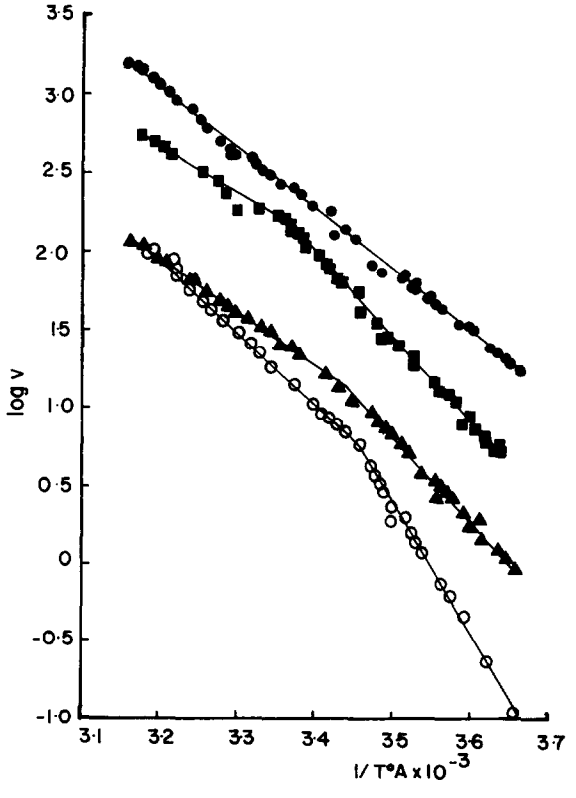

Fig.2. Arrhenius plots of 5′-nucleotidase activity solubilised with Lubrol detergents. Activity is measured as nmol/min/mg protein. Activation energies are given in table 1. Lubrol G (●). Lubrol 17A10 (■). Lubrol N13 (▲). Lubrol 12A9 (○).

Table 1

Detergent	m.p. (°C)	Arrhenius plot (break point °C)	Activation energy (kcal.mol^{-1})		K_m (μM)	Yield (%)
			Above break	Below break		
Native		Biphasic (5) (27.5 ± 0.7)	11.1 ± 0.6	22.6 ± 3.2	20 ± 3	–
Triton X-100[a]	7 (pp)	Biphasic (3) (6.8 ± 1.2)	16.9 ± 0.6	32.0 ± 0.9	25 ± 7	27 ± 3
Deoxycholate	174	Linear (3)	18.5 ± 2.0		25 ± 4	120 ± 15
Lubrol G[b]	−10 (pp)	Linear (3)	18.8 ± 1.6		25 ± 3	315 ± 60
Lubrol 12A9[c]	19	Biphasic (4) (16.1 ± 0.1)	22.6 ± 0.7	33.5 ± 0.8	22 ± 7	294 ± 66
Lubrol N13[d]	20	Biphasic (3) (19.7 ± 0.3)	16.0 ± 2.9	23.2 ± 1.5	24 ± 6	25 ± 22
Lubrol 17A10[e]	23	Biphasic (3) (23 ± 1.3)	15.6 ± 2.2	22.9 ± 2.9	17 ± 1	522 ± 71

[a] Koch Light, catalogue KL4.pp521
[b] ICI data sheet DI385
[c] ICI data sheet DI383, although a change in detergent micelle structure occurring at 16°C has been noted [16]
[d] ICI data sheet DI387
[e] ICI data sheet DI384

pp, pouring point

Numbers in brackets represent the number of determinations

Arrhenius plots of the 5′-nucleotidase activity of preparations solubilized with the Lubrol detergents 12A9, N13 and 17A10, and Triton X-100 were all clearly biphasic with 'break' points occurring at different temperatures (fig.1,2, table 1).

4. Discussion

We have demonstrated that 5′-nucleotidase may be solubilised using a number of different detergents. In most cases the yields of recovered 5′-nucleotidase activity were greater than 100%, indicating that solubilisation led to activation of the enzyme. Such an apparent increase in activity may be achieved by relieving a constraint imposed on the protein by the membrane, or by direct action of the detergent molecules on the enzyme itself. This increase in total 5′-nucleotidase activity was observed using all of the detergents except for Triton X-100 and Lubrol N13. Such an activation may be achieved by the detergents effecting an increase in the V_{max} for the reaction as both the solubilised and native enzymes have very similar K_m values (table 1). The observation that 5′-nucleotidase may be activated by increasing the fluidity of the bilayer [16] presumably by relieving a constraint imposed by the bilayer on the three-dimensional structure of the protein may in part explain the increases in 5′-nucleotidase activity.

The yield of 5′-nucleotidase activity after detergent treatment and subsequent centrifugation will also be determined by the amount of enzyme solubilized by the detergent. The low yields using Triton X-100 and Lubrol N13 may be explained by a low degree of solubilization. Consistent with this is the observation that Triton X-100 enhances enzyme activity when added directly to assays containing plasma membranes [21].

We would like to suggest that another factor modulating the recovered activity is the action of the detergent itself upon the protein. Our justification for such a proposal arises from the form of the Arrhenius plots of the recovered solubilised 5′-nucleotidase activities (fig.1,2, table 1). Arrhenius plots of the 5′-nucleotidase activity of native plasma membranes exhibit a well-defined 'break' at 27.5°C. We have provided evidence that this is due to the occurrence of a lipid phase separation localised in the outer half of

the bilayer and modulating the activity of this membrane-bound enzyme [14,15]. Solubilization effected using any of the detergents clearly removes such a 'break' from the Arrhenius plots (table 1) of 5′-nucleotidase activity. However, the form of the Arrhenius plots of solubilised 5′-nucleotidase activity are not identical (fig.1,2, table 1) and indeed appear to be related to the physical properties of the detergents used to solubilise the enzyme (table 1). A number of the detergents have 'melting' points (or so called 'pouring' points) which lie in the temperature range studied (<1–45°C) and there is good correlation between such melting point temperatures and the temperatures at which break points occur in Arrhenius plots (table 1). The detergents which do not have melting (or pouring) points in this temperature range yielded linear Arrhenius plots (table 1). We suggest that upon solubilization 5′-nucleotidase is liberated from the bilayer as a detergent–(lipo) protein complex, and that the activity of the enzyme may be modulated by the physical properties of the associated detergent. That detergents can modulate the activity of solubilised proteins, and be sufficiently tightly associated to achieve such modulation, may have consequences for attempts to reconstitute membrane functions with proteins solubilised using Lubrol or Triton detergents.

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