In this study of freeze-induced degradation and denaturation, yeast alcohol dehydrogenase, pig liver cytosolic aldehyde dehydrogenase and NADH were chosen as model biological systems.

Methods

Freeze-induced pH changes in frozen biological buffer solutions were measured by the colour change of added universal indicator solution. All buffers, NADH and enzyme samples were frozen in 1 ml plastic vials and defrosted by immersion in 45°C water.

NADH concentrations were determined by enzyme assay [3].

Results and discussion

NADH could not be freeze-stored at -25°C in 50 mM-sodium phosphate buffer (pH range 6-8, 20°C) without serious loss of the nucleotide; however, no losses were seen upon freeze storage in 50 mM-Hepes buffer (pH range 7-8, 20°C). The rate of NADH degradation was greater (approx. 2-fold) in 50 mM- than in 5 mM-sodium phosphate buffer, although NADH values at both buffer concentrations were similar (5% remaining) after the 2 day freezing period. However, NADH stored in liquid nitrogen in 50 mM-sodium phosphate buffer, pH 7.0, retained 89% of its pre-freeze value over a 2 day storage period.

The rate of freeze-induced NADH degradation was not affected by the number of times the sample was thawed and re-frozen. Very little difference was seen in the degradation rate of single freeze samples out that of the multifreeze sample. Thus, it would appear that time spent in the frozen state is the major factor responsible for NADH degradation other than the freezing process itself.

NADH incubated at 4°C at pH 3.7, 5.0, 6.0, 7.0, 7.5 and 8.0 showed considerable degradation at acidic pH. The degradation profile at pH 3.7 at 4°C was very similar (t1/2, 5 h) to that seen with 50 mM-sodium phosphate buffer pH 7.0 upon freezing and storage at -25°C.

From the colour changes observed in frozen mixtures of buffers with universal indicator solution, phosphate buffers were shown to give considerable pH changes upon freezing (-25°C to -195°C, ΔpH 3-6 units), while buffers such as Hepes showed no such changes. In general, buffers made from a mixture of inorganic salts showed greater pH changes than those seen with zwiterionic organic buffers.

Bovine serum albumin retarded the freeze-induced pH changes in phosphate buffers with higher protein concentrations being more effective (7.5 mg of BSA/ml prevented any pH change). Similarly, dimethyl sulphoxide and glycerol retarded the pH changes with 30% of either cryoprotectant preventing any pH change.

On freezing yeast alcohol dehydrogenase and pig liver cytosolic aldehyde dehydrogenase in sodium phosphate and Hepes buffers, pH 7.0, in both cases enzyme losses are much greater in sodium phosphate buffer than Hepes buffer and were greater at -90°C than at -196°C. Sample activity retention showed little dependence on the freezing rate as samples frozen rapidly by immersion in liquid nitrogen and then transferred to -25°C showed similar losses to those frozen slowly from room temperature to -25°C.

Protein freeze storage has, in the past, been more as an art than a science and any rules governing such freeze storage, if indeed any exist, have been rather empirical. The major cause of protein denaturation upon freezing could be due to the development of an acidic pH, although the extent of such denaturation is variable with different proteins [4]. It is suggested that the following steps, if followed progressively, might help to reduce the time spent and the material lost in the usually 'hit and miss art' of protein freeze storage: (1) Where possible, store samples in zwiterionic buffers which show little pH change on freezing (such as Hepes). (2) Storage at liquid nitrogen temperatures. (3) If storage in inorganic buffers cannot be avoided then: (a) determine the pH change of buffer upon freezing by the indicator method; (b) replace one buffer, where possible, with another in which the pH change is smaller (such as potassium phosphate for sodium phosphate); (c) freeze at high protein concentration to prevent pH changes, the indicator method can be used to determine the minimum protein concentration required; (d) use small volumes to allow for rapid freezing and thawing; and (e) use additives: test the cryoprotectant's ability to prevent buffer pH changes by the indicator method; the cryoprotectant action might be protein specific and require a lengthy test procedure.


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Labelling of cysteine proteinases in purified lysosomes

DONNA WILCOX and ROBERT W. MASON

Biochemistry Department, Strangeways Research Laboratory, Worts Causeway, Cambridge CB1 4RN, U.K.

The diazomethane peptide inhibitor, Z-[125I]Tyr-Ala-CHN, has been shown to enter cells and specifically label the lysosomal cysteine proteinases cathepsins L and B [1]. The uptake of similar inhibitors by cells is thought to occur via pinocytosis [2], although passive diffusion of this inhibitor across the membrane cannot be discounted. To determine whether this may occur, we have looked at the ability of this inhibitor to enter isolated lysosomes.

Mouse liver lysosomes were purified according to the method of Yamada et al. [3]. This method involves the pre-swelling of a crude lysosome-mitochondrial fraction with 1 mM-calcium chloride to specifically decrease the density of mitochondria to enhance their separation from lysosomes on a Percoll gradient. The fractions enriched in lysosomal enzyme activity were then used for the study.

Percoll-gradient fractions of lysosomes were labelled for varying lengths of time with 0.1 μM-Z-[125I]Tyr-Ala-CHN. The fractions contained 0.25 M-sucrose at pH 7.4. After labelling, the lysosomes were spun down (10000 g for 10 min) and solubilized using SDS/polyacrylamide-gel electrophoresis (PAGE) sample buffer. Samples were then run on 12.5% (w/v) SDS/PAGE and autoradiographed to visualize the labelled proteins. Immunoprecipitation was performed by solubilization of the lysosomes in buffer containing SDS/Triton X-100 for cathepsin B or sonicated in low-salt buffer for cathepsin L and precipitated as described previously [1]. Three main proteins labelled and were identified as cathepsin L, M, 24000 and cathepsin B, single chain M.
33,000 and light chain Mr, 50,000. Labelling of the three proteins was seen after 10 min, with maximum labelling seen after 1 h. Labelling of cathepsins L and B was only seen in preparations of lysed lysosomes in the presence of diethiothreitol, indicating that a reducing environment is essential for labelling of these enzymes. Therefore, it can be concluded that in these experiments Z-[125I]Tyr-Ala-CHN, labels cathepsins L and B by entering the intact lysosomes.

To assess the effect of Z-[125I]Tyr-Ala-CHN, on the lysis of lysosomes during the labelling, release of sulphatase activity was measured. There was no increase in free sulphatase activity during these experiments, indicating that the inhibitor does not cause lysis of the lysosomal membrane. Previous workers have used osmotic lysis of lysosomes to estimate the ability of carbohydrates, amino acids and small peptides to cross the lysosomal membrane [4, 5]. Our results indicate that although Z-[125I]Tyr-Ala-CHN, does not cause lysis of the membrane, it is able to enter the lysosome, as shown by the labelling of the lysosomal enzymes.

To assess the ability of other cysteine proteinase inhibitors to cross the lysosomal membrane, 'blocking' experiments were performed. E-64 [(3-carboxy-2,3-trans-epoxypropionyl-lysyl)-guanidino] butane, E-64d [(3-ethyl-oxycarbonyl-2,3-trans-epoxypropionyl-lysyl)-guanidino] butane [and Z-[125I]Tyr-Ala-CHN], were incubated, at 10 μM final concentration, with intact lysosomes for 1 h and then 0.1 μM-Z-[125I]Tyr-Ala-CHN, was added and incubated further for 1 h. The labelled proteins were visualized by autoradiography. E-64d and Z-[125I]Tyr-Ala-CHN, completely blocked all labelling by Z-[125I]Tyr-Ala-CHN, whereas E-64 caused only a slight reduction of labelling. The labelling of proteins in the lysed lysosomes was totally blocked by pre-incubation with all three inhibitors. These results show that Z-Tyr-Ala-CHN, and E-64d rapidly cross the lysosomal membrane, whereas E-64 does not. The use of E-64 in cell culture and animal experiments is therefore limited owing to its poor ability to cross biological membranes.

The difference in ability to permeate the membrane may be related to the overall charge of the molecule, rather than the molecular mass. E-64 and E-64d have very similar masses and the main difference between them is their overall charge. E-64 has a strong positive charge at this pH, whereas E-64d has a more neutral charge.

This work has demonstrated an alternative method for investigating the permeability of the lysosomal membrane and will enable the permeability of the lysosomal membrane to other cysteine proteinase inhibitors to be established.

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Co-ordinate diurnal variations in the activities of cholesterol-metabolizing enzymes in the rat mammary gland

JOHN H. SHAND and DAVID W. WEST
Hannah Research Institute, Ayr KA6 5HL, U.K.

The rat mammary gland requires a considerable throughput of cholesterol during lactation. Its requirement is supplied largely (60-70%) from the intestine and liver via plasma lipoprotein particles. In most cells, intracellular cholesterol metabolism is largely governed by the activity of three microsomal regulatory enzymes: hydroxymethylglutaryl-CoA reductase (HMG-CoA reductase, EC 1.1.1.34), as the key enzyme regulating synthesis, acyl-CoA:cholesterol acyltransferase (ACAT, cholesterol acyltransferase, EC 2.3.1.19), which promotes cholesterol ester formation, and cholesterol ester hydrolase (cholesterol esterase, EC 3.1.1.34), which initiates the degradation of cholesterol esters. The activity of HMG-CoA reductase is controlled both by degradation and by a phosphorylation-dephosphorylation mechanism, and is characterized by a diurnal variation both in the total amount of enzyme present and in the proportion of the enzyme that is deactivated by phosphorylation. In the mammary gland this diurnal variation in HMG-CoA reductase activity has a peak at mid-light, the inverse of that observed with the hepatic enzyme [1]. Although the peak activity of the mammary enzyme coincided with the period when cholesterol synthesis in the liver was at its nadir, there was no detectable increase in the amount of cholesterol synthesized within the gland, suggesting that cholesterol stored within the gland supplied the rest. Previous investigations of ACAT and cholesterol ester hydrolase activity within the mammary gland [2, 3] were extended to examine diurnal variations in the activity of these two enzymes and to determine the relationship of such variations in activities to the variation in HMG-CoA reductase activity [1].

The mammary glands of Wistar rats, maintained on a constant lighting regimen (lights on 08.00 to 20.00 h), were freeze-clamped at approx. 3 h intervals and were homogenized (Polytron) in 5 vol. of 250 mM-sucrose, 100 mM-KF, 1 mM-EDTA and 50 mM-K2HPO4, buffer (pH 7.1). After centrifugation (10,000 g) the supernatant was split into two portions and each centrifuged at 100,000 g. The resultant pellets were resuspended in 50 mM-phosphate buffer (pH 7.1) containing 1 mM-EDTA and 100 mM of either KF (fluoride microsomes) or KCl (chloride microsomes) and centrifuged again (100,000 g). The microsomal pellets were stored in the appropriate fluoride or chloride buffers in liquid nitrogen before measurement of ACAT activity in the absence and the presence of exogenously added cholesterol. The activities of both the neutral and the acid cholesterol ester hydrolases present in the chloride microsomes were determined together with the lipoprotein lipase (LPL, EC 3.1.1.34) activity in the same fraction.

The diurnal variation in ACAT activity (pmol of cholesterol olate formed/min per mg of protein) was essentially the same both in the presence and absence of exogenous cholesterol. It attained its peak value (15.66 ± 2.7 pmol/min per mg) at 23.00 h but declined rapidly reaching its low point (7.55 ± 0.8 pmol/min per mg) around 05.00 h. A second smaller peak of activity (14.04 ± 1.5 pmol/min per mg) was centred at 09.30 h but the activity measured at 11.00 h was

Abbreviations used: HMG-CoA reductase, hydroxymethylglutaryl-CoA reductase (EC 1.1.1.34); ACAT, acyl-CoA:cholesterol acyltransferase (EC 2.3.1.19); LPL, lipoprotein lipase (EC 3.1.1.34).