Pure & Appl. Chem., Vol. 64, No. 8, pp. 1055–1060, 1992.
Printed in Great Britain.

Enzyme engineering and its application in lysosomal storage disease

Sofia Mumtaz and **Bimal K. Bachhawat**

Department of Biochemistry, University of Delhi, South Campus, New Delhi-110021.

The efficacy of a number of therapeutically active enzymes in the treatment of inherited lysosomal storage disorders is severely limited by their instability *in vivo*. Modification of the enzymes with dextran enhances their survival in circulation and intracellularly protects them against degredation in the lysosomes. Such enhancement of stability of therapeutically active enzymes would be particularly important in treatment of enzyme deficiency disorders.

INTRODUCTION

A number of inherited metabolic disorders lead to the accumulation of metabolic intermediates in the lysosomes, due to the absence of some key enzyme responsible for their degradation. An early report of enzyme deficiency in metachromatic leukodystrophy came from our laboratory in 1963, and arylsulphatase A was one of the first lysosomal enzymes which was recognised to be deficient in disorders of sphingolipid metabolism (ref. 1). Since then a number of enzymes are now known to be absent in a variety of storage disorders as shown in Table 1.

TABLE 1. The deffects in human lysosomal storage diseases.

Disease	Deficient hydrolase(s)
Tay-Sachs disease, A variant.	β - Hexosaminidase A
Tay-Sachs disease, AB variant.	β - Hexosaminidase activity with GM2 ganglioside as substrate
Pompe's disease	∝ - Glucosidase
Metachromatic leukodystrophy	Arylsulfatase A
GM1 gangliiosidosis	β - Galactosidase
Hurler's disease	∝ - Iduronidase

From Tager, J.M. (1985) Trends Biochem. Sci 10:324.

The treatment of such disorders arising from enzyme deficiencies can be approached in two ways : by correcting the malfunctioning gene or by an exogenous replacement of the missing enzyme. Both the treatments involve the delivery of appropriate molecules (DNA fragments or intact enzymes) to appropriate intracellular sites. In enzyme therapy various approaches have been used to compensate for the defective enzyme. One of them has been organ

transplant, as in the case of Fabry's disease where it has been possible to transplant a normal kidney in a patient (ref. 2). This approach has shown considerable success in alleviating the disorder. Another approach is the transfusion of normal plasma which has been used in the treatment of Fabry's disease and Hurler's syndrome (ref. 3). Though this method had some initial success, its curative effects were short-lived due to a rapid inactivation or removal of enzyme from circulation. Before reaching the site of action these foreign substances provoke adverse immunological reactions, undergo proteolytic degradation by blood enzymes, and are even taken up by RES and nondiseased tissue preventing thereby their delivery to diseased tissue in sufficient amounts.

LIPOSOMES AS A DRUG CARRIER

In the search of an appropriate carrier for such therapeutically active substances it was felt that various problems of enzyme therapy might be circumvented by their entrapment in liposomes. Liposomes, which were first developed by Bangham (ref. 4), are biodegradable lipid vesicles with concentric lipid bilayers alternating with aqueous compartments. Water soluble substances like enzyme can be entrapped in these aqueous compartment.

In vivo experiments have shown that the activity of an enzyme in liposomes remains latent while they are in circulation and much of this enzyme activity is eventually recovered from the hepatic lysosomes, indicating that the liposomally encapsulated enzyme is protected from the environment. Another advantage in the use of liposomes is that they can be targetted to the diseased tissue. Kinsky suggested that liposomes can be prepared by incorporating glycolipids such as cerebrosides into the matrix with the carbohydrate moiety of the glycolipids exposed on the surface of the liposomes (ref. 5). This led us to suggest that modification of the liposomes by incorporating various glycolipids having nonreducing terminal sugars would facilitate the targetting of the liposomes to different tissues carrying different terminal sugar specific receptors (ref. 6). Ghosh *et al* showed that liposomes containing asialo GM1 ganglioside, when administered intravenously accumulated in the liver. The observed uptake was dependent on the density of the galactose residues on the liposomal surface, and was inhibited by competition with asialofetuin (ref. 7). They further observed that β - gal liposomes accumulated preferentially in the hepatocytes as compared to α - gal liposomes (ref. 8,9) indicating that the hepatic receptors are specific for β - gal containing liposomes.

It has been shown that when such liposomes with the entrapped enzyme are administered intravenously into mice. They are taken up predominently by the livers of the animals. Subcellular fractionation shows a considerable proportion of this to be in the lysosomes of the cells (ref. 10). This is of significance as quite a large number of inherited enzyme disorders arise from the deficiency of lysosomal acid hydrolases. All these factors suggested that liposomes would be ideal carriers for therapeutic enzymes.

Gregoriadis and Buckland tested the efficacy of liposomes as enzyme carriers in a model for storage diseases. When Chinese hamster fibroblasts were exposed to sucrose, there was an accumulation of sucrose within their lysosomes due to the absence of invertase in these cells. Subsequent exposure of these cells to liposomally encapsulatd invertase led to the degradation of the stored sucrose indicating that invertase was incorporated

into the cells (ref. 11). In another study, Colley and Ryman developed a model storage disorder in rat liver by accumulating dextran. In the absence of the enzyme 'dextranase', dextran is not degraded in mice. However, administration of liposomally encapsulated dextrance led to a significant degredation of stored dextran (ref. 12). Liposomes, therefore, appear to be promising as vehicles for the administration of enzymes.

Gregoriadis and Ryman observed that although liposomally entrapped amyloglucosidase could be selectively delivered to the liver lysosomes, but once there it is very rapidly catabolised (ref. 13). This is true for other enzymes proteins as well. The rapid degradation of the administered enzyme severely limits its therapeutic potential and thereby the efficacy of enzyme therapy in controlling such disorders. Work therefore started in several laboratories to find ways of enhancing the stability of the administered enzyme in the lysosomes. In order to do so successfully it is first important to understand the nature of lysosomal hydrolases, which despite being proteinacious in nature are remarkably stable in the acidic milieu of the lysosomes. These enzymes have some properties in common : (a) They have in general an acid pH optimum, (b) they are generally resistant to autolysis and (e) they are glycoproteins. The glycoprotein nature of the lysosomal hydrolases and their resistance to autolysis lead us to investigate the importance of the oligosaccharide part of these glycoprotein enzymes. Work done in our as well as in other laboratories showed that removal of the terminal sialic acid does not impair the catalytic activity of the enzyme (ref. 14). However, removal of the terminal sugar lead to a markedly reduced survival time in the circulaton indicating that it had a role to play in the *in vivo* circulatory life of the enzyme. The glycoproteinic nature of the lysosomal enzymes as well as their resistance to autolysis led us to suggest that it might be possible to prolong the *in vivo* survival of enzymes by their covalent modification with polysaccharide (ref. 6).

EFFECT OF COVALENT MODIFICATION OF ENZYMES WITH PEG AND DEXTRAN

With a view to enhance the stability of enzymes *in vivo*, various investigators covalently modified a number of enzymes with hydrophilic polymers such as polyethylene glycol (PEG) and dextran (for review ref. 15). They have shown that PEG and dextran reduce the immunogenicity of a number of enzymes with a minimally deleterious effect on their catalytic functions (ref. 16-20). This is particularly important where repeated treatment with the enzyme is required over a period of time. Streptokinase, which promotes autolysis by activation of the fibranolytic system of the human blood and is clinically used in the treatment of thrombic states in man, is one such enzyme (ref. 21). Being an enzyme of bacterial origin, it is strongly antigenic. Koide *et al* reported a complete loss of antigenicity with little or no effect on the activity of this enzyme after its modification with PEG (ref. 22).

Such modified enzymes also exhibit enhanced circulatory life as compared to the native enzyme (ref. 23-25). Various factors have been evoked to account for this increase in circulatory life. These include loss of antigenicity, increase in size of small peptides and hence a decreased clearance by glomerular filteration in the kidney (ref. 25). The hydrophilic coating also prevents the binding to and removal of the enzyme by "opsonins", which are known to have particular affinity towards the hydrophobic regions of the molecule (ref. 26).

In vitro studies have shown that the modified proteins exhibit enhanced resistance to proteolytic degradation (ref. 18,27). This could well be responsible for the increased circulatory life. Such modified enzymes are increasingly finding clinical use in the treatment of various deficiency disorders (ref. 28,29). Although enhanced stability and prolonged life span of enzymes in circulation have been demonstrated by a number of investigators, no reports are available on the stability of enzymes inside the cells with particular reference to the lysosomes. We therefore decided to covalently modify an enzyme with a non-degradable sugar polymer in order to mimic the glycoproteinic nature of lysosomal hydrolases and study its stability in the cells.

Horse raddish peroxidase (HRP) was chosen for this study and it was covalently modified by conjugation with dextran. Preliminary studies indicated that the modified HRP had an increased circulatory life as compared to the native enzyme. Intracellularly the dextran-HRP conjugate appeared to be more stable in comparison with the native HRP when equal amounts of both were administered intravenously into swiss albino mice (Table 2). However no definitive conclusions could be derived from these studies as the rate of uptake of HRP into the liver was considerably faster than that of dextran-HRP.

TABLE 2. Comparison of the percentage of various formulations of HRP remaining in the total liver and the mitochondrial/lysosomal fraction 24 hours after administration.

Formulation used	Percentage enzyme activity	
	Homogenate	M/L fraction
Free HRP	0.0	0.0
Liposomally encapsulated HRP	6.0	5.0
dextran-HRP	9.0	10.0
Liposomally encapslated dextran-HRP	39.0	75.0

Data are expressed as percentage of HRP concentration in the liver or mitochondrial/lysosomal fraction 24 hours after administration when the amount taken up within 30 minutes is considered to be 100%. Each value represents the mean of three experiments.

In order to ensure a comparable rate of uptake of HRP and dextran-HRP by the liver both were encapsulated into liposomes and then administered intravenously. The amounts of HRP or dextran-HRP present in the liver and the mitochondrial/lysosomal fractions were studied at varying intervals of time after their administration. Maximal uptake was observed 30 minutes after administration for both HRP and dextran-HRP. When this amount was considered to be 100% and degradation measured thereafter, it was observed that almost no liposomally encapsulated HRP could be detected in the liver and the subcellular fractions 24 hrs after administration (Table 2). This is in comparison to almost 40% of the liposomally delivered dextran-HRP conjugate present in the liver and 75% present in the mitochondrial/lysosomal fraction after the same period of time (Table 2).

It appears that covalent modification of HRP with, an inert sugar polymer, dextran is preventing its degradation by the lysosomal hydrolases. This may be due to protection against proteolytic degradation as has already been shown in *in vitro* studies (ref. 18,27). The enhanced stability of enzymes and use of liposomes for targetting would be of great significance in the treatment of a number inherited enzyme deficiency disorders. This is the first time that a method of enhancing the intracellular stability of enzymes has been reported and it suggests a new approach to enzyme therapy for the treatment of storage disorders (Mumtaz S. and Bachhawat B.K., manuscript submitted for publication).

Acknowledgements

Sofia Mumtaz is a Senior Research Fellow of the University Grants Commission. We are grateful to Prof. U.N. Singh for critically analysing the manuscript. We are thankful to Ms. Poonam for preparation of the manuscript on computer. Financial assistance by the Department of Biotechnology is gratefully acknowledged.

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