New Protein Structure-Function Technology and Applications

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ABSTRACT

Recent advances in protein structure technology such as affinity chromatography, high performance liquid chromatography (for protein and peptide isolation) and microsequencing have provided new opportunities for answering fundamental questions as well as providing new applications. For example, it has been recognized for many years that aging causes the accumulation of "abnormal" proteins in cells. The amount of these aged proteins in human cells is exceedingly small, and only through the use of microstructural analyses has it been possible to explore the properties of aged proteins. Such studies suggest that aged cells have a decreased ability to catabolize covalently modified proteins. High-sensitivity structural analyses also provide the opportunity to study the molecular basis of genetic diseases with small amounts of tissue or blood. Examples of the aplication of these new technologies include isolation of enzymes for therapeutic purposes such as digestion of herniated spinal discs and the debridement of burns and ulcers. The use of microencapsulated proteins and matrix-bound enzymes also has provided new application opportunities. For example, covalently bound lactase has been found to have bio-adhesive properties in the intestine and may be used for treatment of lactase insufficiency in persons who cannot tolerate milk or milk products.

INTRODUCTION

Wilson (1) has reviewed recent advances in the methods and technology for protein structural analysis, and Feeney (2) has summarized the current status of protein chemical modifications. In this review we present examples of how these advances in protein structural analysis and chemical modification have made possible both basic and applied investigations which previously were not possible. We will deal first with basic studies, then examples of applied studies.

RESULTS AND DISCUSSION

The Molecular Basis of the Accumulation of Abnormal Proteins During Aging

It has long been recognized that changes in proteins seem to occur with age. The appearance of the brown-age pigments of lipofuscin in skin, and the changes in the texture of skin due to structural changes in collagen, are just two examples. More recently, studies in animals, man and cells grown in tissue culture have indicated a general pattern for the accumulation of "abnormal" proteins during aging. Enzymes in aging cells seem to be: (a) more unstable, (b) present in lower levels of activity, and in many cases (c) more negatively charged. It has been generally postulated that the accumulation of such "abnormal" proteins might interfere with normal metabolism, thereby compromising the ability of the cell to cope with environmental stresses (i.e., decrease its ability to survive). Although several theories as to the molecular basis of this process have been proposed, it is clear that only when the differences in the structures of "young" and "senescent" proteins are known can the mechanism(s) for accumulation of abnormal proteins be eludicated.

Triosephosphate isomerase TPI (EC 5.3.1.1) is an enzyme which may provide a "marker" allowing this problem to be addressed by means of the new technology of high-sensitivity protein structural analyses. TPI occurs as a single, very stable enzyme in cells from young persons. However, with age, additional more negatively charged electrophoretic forms of the enzyme are found. This is the case in lymphocytes from aging humans, in "old" erythrocytes and in aging skin fibroblasts. Table I shows the accumulation of the labile, acidic isozymes in aging skin fibroblasts. While only a small amount of the "senescent enzyme" is present in fibroblasts from young individuals, cells from old persons contain considerably more of the "senescent isozyme." This same pattern exists for fibroblasts from young donors which are aged in vitro through extensive population doublings. Moreover, the same accumulation is observed in fibroblasts from individuals with premature aging diseases such as Progeria and Werner's syndrome.

Using techniques similar to those described by Wilson (1), we were able to isolate to homogeneity the "young" and "senescent" forms of TPI from human cells (3,4) and subject them to high-sensitivity structural analyses (5-8). The entire amino acid sequence of the enzymes was elucidated (8). The only differences found between the "young" and "old" forms of TPI were in the deamidation of two specific asparagine residues. In the "young" form of the enzyme Asn-15 and Asn-71 are found, but with age these are deamidated to aspartic acid residues.

A model of the dimeric enzyme based on a comparison with the X-ray data of the enzyme from other sources has been constructed (9). The locations of these specific asparagines are in the subunit-subunit contact sites and are juxtaposed to each other. These specific deamidations result in four new negative charges in the contact site which cause destabilization and dissociation of the dimer. This seems to account for the decreased stability of these more acidic isozymes. Furthermore, the deamidated forms of the enzyme are much more susceptible to proteolytic hydrolysis. It appears that deamidation is the first step in the normal catabolism of the enzyme, and that in normal young cells the deamidated isozymes are rapidly hydrolyzed by proteases. In senescent cells, the deamidations occur as usual but the failure to degrade these isozymes results in their accumulation (10).

TABLE I

Accumulation of Deamidated Triosephosphate Isomerase in Aging Cells[a]

Fibroblasts	Percent deamidated TPI
Normal young[b]	0- 3
Normal old[c]	8-15
in vitro Aged[d]	11-18
Progeria	30-40
Werner's	18-25

[a]The relative amounts of non-deamidated and deamidated TPI were determined by electrophoresis on polyacrylamide gels followed by specific activity staining as described previously (9).

[b]18-30 years of age donors, early passages 3-10.

[c]79-92 years of age donors, early passages 3-14.

[d]22-35 years of age donors, late passages (28-38).

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In order to test this hypothesis, we conducted several in vivo experiments aimed at increasing and decreasing levels of proteolysis. Table II shows the effects of starvation of human fibroblasts by serum deprivation. Starvation is known to increase proteolysis markedly. Indeed, the levels of the deamidated TPI isozymes decrease as proteolysis is stimulated by starvation. Conversely, the inhibition of proteolysis by insulin addition causes an increase in the relative levels of deamidated TPI (Table III).

While detailed structural-functional studies on other "abnormal" enzymes in aged cells have not yet been completed, a similar pattern seems to be emerging. Wulf and Cutler (11) have made similar observations in animal studies. They measured the levels of "unstable" glucose 6-phosphate dehydrogenase (G6PD) in tissues of young and old rats during fed and starved conditions. Their studies showed that in tissues from young animals only a very small amount of unstable G6PD was present. However, upon aging, elevated levels of the unstable enzyme accumulated. In addition, upon starvation of the animals the levels of unstable enzyme were markedly reduced.

Therefore, it is clear that the ability to conduct structural studies on proteins at the picomole level has provided the opportunity to study changes in proteins which occur during the aging process. Obviously, future studies of this type should be particularly interesting in further understanding the molecular processes whereby these "abnormal" proteins accumulate in aging cells.

A second example where the new technology in protein structure has made impact is in the elucidation of precise mutations responsible for genetic diseases. Figure 1 outlines a three-phase program for studying several genetic defects of enzymes of carbohydrate metabolism. These genetic diseases result in severe hemolytic anemias in children. Phase I is aimed at the identification of the particular enzyme which is defective. Very small amounts of blood (e.g., a single drop) can be used to measure the levels of each of the suspect enzymes and to examine their electrophoretic properties. While this type of diagnosis is an important first step to identifying the abnormal enzyme, Phases II and III are essential for the precise identification of these mutations. Thus, mechanisms for protein structural studies at the sub-nanomole level are essential. Glucosephosphate isomerase (GPI) deficiency is the third most common enzyme defect of erythrocytes and exhibits a wide range of mutations. This enzyme contains a relatively large subunit of 65,000 molecular weight. It is a very basic protein with a large number of lysine and arginine residues. Tryptic digestion results in a very complex mixture of peptides. Likewise, cleavage at the 13 methionine residues with CNBr poses similar problems. On the other hand, specific chemical cleavage at the three cysteine residues with 2-nitro-5 thiocyanobenzoic acid results in only four peptides which were easily isolated (12). After cleavage of GPI at the cysteine peptide bonds, essentially no native enzyme was found and virtually no overlap peptides were obtained. Thus, this method allowed the quantitative, specific cleavage of the protein, and the alignment of the peptides for the further identification of the sites of the mutations.

A third example of enzymes in applied technology is the use of proteases to digest blood clots (13), treatment of ulcers (14), thrombosis (15), and the chemonucleolysis of herniated spinal discs (16). The proteolytic enzyme, chymopapain, is isolated from the papaya fruit and is ideally suited to digest the mucoprotein which becomes exposed and swollen in the ruptured spinal disc. The injection of this enzyme causes digestion of the nucleus pulposus of the herniated disc with less complications, costs and risks than surgery. Clearly, the medical use of other enzymes as similar therapeutic aids will be forthcoming.

Finally, we present an example of the use of enzyme therapy in the treatment of genetic disorders. Most adults in the world (the notable exceptions being some areas of Western Europe and the U.S.) suffer from deficiency in the enzyme lactase. This enzyme catalyzes the hydrolysis of the disaccharide, lactose, into the monosaccharides glucose and galactose, permitting the absorption of the monosaccharides. This enzyme is located on the surface of the brush border cells of the small intestine. However, in some infants and in many adults, low levels of the enzyme result in failure to hydrolyze the disaccharide, and the consequent fermentation of the sugar by bacterial flora results in severe diarrhea, vomiting and the inability to metabolize milk and dairy products. For several years attempts have been made to

TABLE II

Effect of Starvation on Levels of Deamidated Triosephosphate Isomerase

Starvation period[a] (days)	Percent deamidated TPI
0	23
2	14
4	12
10	3

[a]Human skin fibroblasts were grown in the presence of complete media plus 10% fetal bovine serum (i.e., starvation period 0). Cells were then placed in fresh media without fetal bovine serum. Levels of deamidated TPI were measured at the indicated periods by methods previously reported (10).

TABLE III

Effect of Insulin on Levels of Deamidated Triosephosphate Isomerase

Insulin (units/ml)	Percent deamidated TPI
0	9
0.05	12
0.10	13
0.20	14

Human skin fibroblasts were first deprived of fetal bovine serum for two days. The cells were then treated with media containing the indicated levels of insulin for a 3-hr period while being incubated at 37 C. Cells were then harvested and assayed as described previously (10).

PHASE I

1. Electrophoretic profile
2. Enzyme activity levels

PHASE II

1. Purification of enzyme by affinity chromatography
2. Resolution of varients in heterozygotes
3. Catalytic properties
4. Stability properties
5. Molecular weight
6. Immunological properties

PHASE III

1. Fragmentation into peptides
2. HPLC isolation of peptides
3. Microsequence analysis of variant peptide(s)

FIG. 1. Genetic screening program.

TABLE IV

Stability of Lactase in Duodenal Fluid[a]

Lactase	Conditions	Time (min)	Activity (%)
Yeast	Tris pH 8.5	0	100
		20	79
		40	75
		60	70
Yeast	Duodenal fluid	0	100
		20	10
		40	4
		60	0
Fungal	Tris pH 8.5	0	100
		20	93
		40	96
		60	94
Fungal	Duodenal fluid	0	100
		20	90
		40	92
		60	92

[a]Yeast or fungal lactase was incubated at 37 C in either 50 mM Tris (HCl) pH 8.5, or in human duodenal fluid. Samples were removed at the indicated intervals and assayed for lactase activity.

TABLE V

Comparison of Retention of Soluble and Matrix-Bound Lactase in Gastrointestine[a]

Sample	Conditions	Stomach	Small intestine	Large intestine
Soluble	160 min post injection	26.7	15.2	58.0
Matrix-bound	160 min post injection	80.3	19.4	0.3
Soluble	20 hr post injection	17.6	3.8	12.2
Matrix-bound	20 hr post injection	29.4	67.9	2.6
Soluble	26 hr post oral administration	18	9	16
Matrix-bound	26 hr post oral administration	16	10	74

[a]Fungal lactase was radiolabeled in 50 mM sodium phosphate pH 7.5 with 200 μg chloramine T and 1 mCi NaI^{125}. Samples of either the soluble enzyme or the enzyme bound to Sepharose 4B were administered to the stomach of rats either by direct injection or via a stomach tube. After the indicated intervals the animals were killed and the entire gastrointestine removed and counted in a gamma ray spectrometer.

supply this enzyme to the intestine so that it could assist in hydrolysis of the milk sugar. Table IV shows that when yeast lactase is exposed to duodenal fluid it rapidly loses catalytic activity. However, lactase isolated from fungi is active and very stable in the intestinal environment. Moreover, the covalent coupling of the enzyme to a solid matrix (Sepharose 4B) results in markedly improved bioadhesive properties. When the matrix-bound enzyme is introduced into the stomach either via a stomach tube or by injection, it is retained in the gut for much longer periods than the free, soluble enzyme (Table V). These observations suggest that the matrix-bound enzyme may be better suited for treatment of lactase deficiency disease and that the increased bioadhesive properties of matrix-bound enzymes may be useful for treatment of other digestive disorders.

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