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Isozymes

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A great many different enzymes have been found to exist in more than one molecular form as isozymes or isoenzymes. Indeed it is apparent that the phenomenon of isozyme formation is very common throughout a large number of species, including man.

The principal technique which has been used in the study of isozymes is the method of zone electrophoresis, usually in starch or acrylamide gels, together with a specific sensitive stain for the detection of the isozymes after electrophoresis. At first the staining methods available were limited and simple but new methods have been steadily developed and now probably more than a 100 different enzymes and their isozymes can be studied by zone electrophoresis. Good separations can usually be accomplished relatively simply with such materials as crude tissue homogenates, red cell lysates, plasma, urine, saliva, etc, which do not require extensive treatment before analysis. Furthermore, zone electrophoresis methods can also be adapted to provide information about characteristics such as substrate specificity, pH activity, thermal stability, inhibition, reactive groups such as sulphhydryl groups, as well as information about the electrophoretic properties of the isozymes of a particular enzyme. And all of these properties may be important in assessing the molecular characteristics of a particular enzyme and its isozymes. Other separation techniques, in particular gel filtration chromatography and ion exchange chromatography, may be useful, especially for the purification and detailed biochemical analysis of isozymes but in general can be regarded as adjuncts to the electrophoretic methods of analysis. Even so, it should be emphasized that isozymes do not necessarily differ in their electrical charge and therefore do not always separate on electrophoresis. Alternative general methods for their separation, not dependent on charge differences, would be extremely useful and might reveal a whole new series of isozymes which are not demonstrable by current methods.

However, it has become clear simply by the electrophoretic methods of analysis, that isozymes are complex and may be generated in a variety of ways. Furthermore, even in the case of one particular enzyme, more than one kind of cause may often be operating to produce the isozymes which are actually observed. One method of classification (Harris, 1969) divides isozymes into three categories according to how they are generated and this system will be adopted in the present paper. Examples of each of the three major types of isozyme will be discussed.

Secondary Isozymes

These are isozymes which arise by secondary changes in the structure of an enzyme protein subsequent to its primary synthesis on the ribosomes. They are sometimes referred to as posttranslational isozymes and they may arise *in vivo* within the cells and/or *in vitro* during the storage or handling of specimens.

ISOZYME PATTERNS IN YOUNG AND OLD RED CELLS AND IN OTHER TISSUES

An example of secondary isozyme formation *in vivo* is provided by the enzyme human nucleoside phosphorylase (NP) (Edwards, Hopkinson, and Harris, 1971; Turner, Fisher, and Harris, 1971). When red cell lysates are examined electrophoretically for nucleoside phosphorylase (NP) a series of seven or eight NP isozymes are seen. Red cells have, of course, a relatively long life span (> 100 days) and lose their nuclei and stop enzyme synthesis at an early stage. A sample of red cells taken from a normal individual therefore consists of cells of a wide range of different ages. If one takes advantage of their different specific gravities and separates the cells by density gradient centrifugation, it is possible to examine and compare the isozyme patterns in red cells of different ages. When this is done it is found that the faster moving, ie, more anodal, NP isozymes are relatively more prominent in the older cell fraction, and the slower moving, ie, less anodal, NP isozymes are relatively more prominent in the younger cell fraction and the least anodal isozyme is the most prominent. A similar effect is observed when whole red cell samples from normal individuals and from individuals with chronic haemolytic anaemia, eg, thalassaemia, are compared. In the

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reticulocyte-rich specimens from the haemolytic cases, the slower moving isozymes are relatively much more active than in normal individuals, and the faster moving isozymes are relatively weaker. Thus there appears to be a direct correlation between the relative intensities of the various NP isozymes and the 'age' of the red cell. In other tissues such as liver and kidney, similar NP isozyme patterns are seen except that the slower moving zones predominate, the slowest are the most active and the others diminish in activity with increasing electrophoretic mobility. The cells in such tissues no doubt vary in age but they are, in contrast to the peripheral red cell, nucleated and presumably capable of fairly continuous enzyme synthesis. In rapidly dividing cells, such as tissue culture fibroblasts and lymphoid cells in culture, the slowest NP isozyme accounts for virtually all the enzyme activity, though the next one or two isozymes in the series may also be observed as minor zones. These results suggest that the slowest moving, ie, least anodal, isozyme is the primary form of nucleoside phosphorylase synthesized in all cells and that the multiple series of discrete, more rapidly moving isozymes, observed in varying proportions in different tissues, are secondary isozymes that are generated in a stepwise manner from the primary form and from one another as the enzyme protein ages *in vivo*.

Electrophoretic studies of the isozyme patterns of several other enzymes, for example, phosphoglucomutase (Monn, 1969a), pyruvate kinase (Paglia and Valentine, 1970), and pyrophosphatase (Fisher, Turner, Dorkin, and Harris, 1974), in young and old red cells have shown that secondary isozyme formation, similar to that observed in the case of nucleoside phosphorylase, is a very common phenomenon. In most cases it seems that the least anodal component of a set of isozymes is the primary form synthesized and that the others are subsequently derived from this by some intracellular stepwise process.

As yet little is known in most cases about the nature of the structural alterations that may be involved in this generation of secondary isozymes. One suggestion from work on rabbit muscle aldolase (Midelfort and Mehler, 1972) and human carbonic anhydrase (Funakoshi and Deutsch, 1969) is that the loss of amide groups from asparagine or glutamine residues in the enzyme protein could lead to the generation of multiple secondary isozymes. Another possible explanation coming, for example, from work on human salivary amylase (Keller, Kauffman, Allan, and Williams, 1971; Karn, Shulkin, Merritt, and Newell, 1973), is that the addition of carbohydrate side chains to reactive residues on the primary isozyme may lead to secondary forms. Varying degrees of acetylation, methylation, sialation, or phosphorylation of an enzyme protein may also be envisaged as causes of secondary isozyme formation; indeed there is evidence from several enzymes (eg, Segal, 1973) that such reactions may be reversible and physiologically important in regulating metabolism. Cleavage of a polypeptide chain by a proteolytic enzyme is another source of secondary isozymes; for example, human pancreatic carboxypeptidase, A_1, appears to be the primary isozyme produced by activation of procarboxypeptidase A by trypsin. A second isozyme, carboxypeptidase A_2, is produced subsequently by the action of an endopeptidase (trypsin or chymotrypsin) on the A_1 isozyme (Hadorn and Silberberg, 1968).

CONFORMATIONAL ISOZYMES

Another possibility is that secondary isozymes do not differ from each other in their chemical structure but differ in their three-dimensional arrangement. Such conformational isozymes ('conformers') would be expected to show similar enzymatic activity but because of the particular spatial arrangement of their constituent amino acids in each conformation would differ in other properties such as electrophoretic or chromatographic behaviour. They should also be interconvertible by appropriate chemical treatment. So far no completely certain examples of conformational isozymes have been identified but there are some examples of multiple isozymes (eg, Kaplan, 1968; Fisher and Harris, 1971a and b) where the idea of 'conformers' is strongly supported by the experimental evidence.

SULPHYDRYL REACTIONS

Secondary isozymes can also be generated by the reaction of the sulphydryl (-SH) group of cysteine residues in an enzyme protein with other substances such as oxidized glutathione. Such reactions have been demonstrated *in vitro* for red cell acid phosphatase (Bottini and Modiano, 1964; Fisher and Harris, 1969), adenosine deaminase (Spencer, Hopkinson, and Harris, 1968; Hopkinson and Harris, 1969a), and peptidases C and D (Lewis and Harris, 1967, 1969), and appear to be the cause in these cases for the change in isozyme pattern observed in haemolysates on storage. The occurrence of such changes may be confusing in genetical studies unless their cause is recognized.

A particularly striking example of an alteration in isozyme patterns due to sulphydryl reactions is provided by red cell adenosine deaminase (ADA). When freshly prepared haemolysates are examined by electrophoresis the majority of examples show a single main izozyme and one or two weakly active

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more anodal isozymes. If the red cell lysates are stored for a few days in a refrigerator and then re-examined the isozyme pattern is found to be altered. The slowest moving zone becomes progressively weaker while the other, previously minor zones, increase in intensity. After about 10 days' storage the slowest zone is almost undetectable whereas the intermediate zone has about the same intensity as originally shown by the slowest zone. Also an extra, even more anodal, isozyme becomes visible. Thus the final appearance of the isozyme pattern in the stored haemolysate resembles the original pattern except that each of the zones appears to migrate more rapidly towards the anode. When haemolysates are stored it is known that oxidized glutathione accumulates at the expense of reduced glutathione, and this change appears to be responsible for the generation of secondary changes in the ADA isozyme patterns. The deliberate addition of oxidized glutathione to freshly lysed red cells produces the same change in the ADA isozyme pattern, while the addition of mercaptoethanol or reduced glutathione reverses both the effects of storage and the effects of added oxidized glutathione. The oxidized glutathione evidently reacts with a free sulphydryl on each ADA isozyme to form a mixed disulphide. Thus each isozyme acquires an extra negative charge and hence a greater anodal electrophoretic mobility. Similar effects can be demonstrated by the use of other thiol reagents such as dithiothreitol, iodoacetic acid, iodoacetamide, maleate, n-ethylmaleimide, cysteamine, and p-chloromercuribenzoate. With each reagent a reaction product is formed with characteristic electrophoretic mobility which could be predicted from the known properties of the particular reagent. In general, the results obtained were entirely compatible with the idea that each ADA isozyme contains free sulphydryl groups.

The methods used to investigate the ADA isozymes have proved to be generally useful in the investigation of other isozymes. The application of particular interest has been the use of 'thiol' reagents for the detection and characterization of inherited variants due to mutations which have resulted in the substitution of a cysteine residue for some other residue at an exposed part of an enzyme protein. Two probable examples of this have been identified, one in a particular rare peptidase variant (Sinha and Hopkinson, 1969) and the other in a rare phosphohexose isomerase variant (Hopkinson, 1970).

Isozymes due to Multiple Alleles

The occurrence of more than one allele at a particular gene locus is another cause of isozymes. Variant alleles may be relatively rare in the general population or they may be quite common and give rise to genetic polymorphism. Each allele codes for a structurally distinct polypeptide chain; thus the primary structure of the enzyme protein may vary from individual to individual according to the particular alleles which are carried. If an individual is heterozygous for two such alleles, eg, A^1 and A^2 which determine α^1 and α^2 polypeptides respectively, at a given enzyme locus, then more than one molecular form of that enzyme is likely to occur, though the precise number of isozymes observed in such heterozygotes will depend on the basic subunit structure of the enzyme.

SUBUNIT STRUCTURE OF ENZYMES

If the enzyme is a monomer, there will be two isozymes in the heterozygote, which will correspond to the separate α^1 and α^2 polypeptides. If it is a dimeric protein there will be three isozymes: two of these will have subunit structures $\alpha^1\alpha^1$ and $\alpha^2\alpha^2$ and will correspond with the forms seen separately in the respective homozygotes; the third will be a hybrid isozyme, $\alpha^1\alpha^2$, made up of both types of subunit. It will have intermediate electrophoretic mobility and will be peculiar to the heterozygotes. If the enzyme is a trimer, four isozymes will occur in heterozygotes: the two outer isozymes, α^1_3 and α^2_3, will represent the homozygous products of the two separate alleles and the two middle isozymes will be hybrids, $\alpha^1_2\alpha^2_1$ and $\alpha^1_1\alpha^2_2$, containing both types of subunit but in different combinations. If the enzyme is a tetramer, then five isozymes will occur in the heterozygotes (α^1_4, $\alpha^1_3\alpha^2_1$, $\alpha^1_2\alpha^2_2$, $\alpha^1_1\alpha^2_3$, α^2_4) and three of these will be hybrids. Examples of all these types of isozyme patterns in heterozygotes have been identified in electrophoretic studies on different enzymes (Hopkinson and Harris, 1971) although in practice the patterns may be complicated by the occurrence of secondary isozymes of the kind discussed in the previous section.

It is interesting to note that the present evidence suggests that most enzymes are made up of more than one polypeptide subunit. A recent tabulation (Harris, H., personal communication) of the data on some 50 different human gene loci which determine enzyme structure indicates that only about a quarter ($\sim 26\%$) of our enzymes are monomers: the majority are dimers ($\sim 52\%$) or tetramers ($\sim 20\%$). Only one example of a trimeric enzyme has been found so far, ie, nucleoside phosphorylase (Edwards *et al*, 1971). Thus trimers appear to be rather rare and monomers appear to be somewhat less frequent than one might have supposed.

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ASYMMETRICAL ISOZYME PATTERNS

If the two different polypeptides coded by the pair of alleles in a heterozygote have similar kinetic properties and stabilities and are synthesized at the same rates, then the electrophoretic patterns of the principal isozymes in such individuals should be symmetrical in appearance. In the case of a monomeric enzyme the activity ratios will be 1:1 for the two isozymes, in a dimer the activity ratios will be 1:2:1 for the three isozymes, in a trimer 1:3:3:1, and for tetramers 1:4:6:4:1. Many of the isozyme patterns actually observed in heterozygotes over a wide range of different enzymes do show such symmetry. However, in some heterozygotes a distinctly asymmetrical pattern is observed which suggests that in these cases one allele is contributing significantly less than the other to the total enzyme activity.

In most cases the functional significance of these low activity alleles is obscure but there are some examples where they are important clinically as mutant alleles which produce gross enzyme deficiency with pathological results. A clear instance is found in work on the isozymes of phosphohexose isomerase (Detter, Ways, Giblett, Baughan, Hopkinson, Povey, and Harris, 1968). Several rare electrophoretic variants of phosphohexose isomerase (PHI) occur, and family studies show that most individuals with a variant phenotype, eg, PHI 2-1, 3-1, 4-1, etc, are heterozygous for a common allele PHI^1 and for one or other of a series of rare alleles $PHI^{2,3,4}$ etc. (In this discussion the phenotype is denoted PHI and the genotype is italicized, *PHI*.) Most people are homozygous for the common PHI^1 allele and exhibit the PHI 1 pattern. The enzyme is a dimer and in most of the heterozygotes a symmetrical triple banded isozyme pattern is observed. However, some heterozygotes, eg, PHI 9-1 and PHI 10-1, show asymmetrical patterns, suggesting that the contribution of these variant alleles to the total enzyme activity is significantly less than that of the common PHI^1 allele. Indeed, from the appearance of these isozyme patterns alone it would appear that individuals homozygous for PHI^9 or PHI^{10} or compound heterozygotes for these two alleles would exhibit a severe deficiency of PHI activity.

Examination of the PHI isozyme patterns in red cells from several different patients with chronic haemolytic anaemia associated with severe deficiency of red cell PHI activity has shown that this is a valid conclusion and also revealed several interesting examples of genetic heterogeneity due to multiple allelism of the sort discussed in this symposium by Professor Harris. One case (Baughan, Valentine, Paglia, Ways, Simons, and DeMarsh, 1968; Detter, Ways, Giblett, Baughan, Hopkinson, Povey, and Harris, 1968) was found to be heterozygous for the two electrophoretically distinct rare alleles PHI^9 and PHI^{10}; the mother was phenotype PHI 9-1 and the father was PHI 10-1. In another family (Tariverdian *et al*, 1970) two anaemic children were found to be homozygous for the rare PHI^9 allele, and both parents were of the rare phenotype PHI 9-1. The anaemic children in a third family (Paglia, Holland, Baughan, and Valentine, 1969) exhibited a single weak isozyme just slightly less cathodal than the usual PHI^1 isozyme and were presumably homozygous for another rare electrophoretic allele. The isozyme patterns in the presumably heterozygous parents were not distinguishably different electrophoretically from the usual PHI 1 pattern. This could be due to the technical difficulties of separating isozymes of very similar electrophoretic mobilities or might be due to the variant isozymes being more labile than the PHI^1 isozyme and thus undetectable in these heterozygotes. More recently several new cases of PHI deficiency have been reported (eg, Blume, Hryniuk, Powers, Trinidad, West, and Beutler, 1972; Nakashima, Miwa, Oda, Oda, Matsumoto, Fukumoto, and Yamada, 1973) and the isozyme studies have revealed yet further genetic heterogeneity.

Isozymes due to Multiple Gene Loci

Some enzymes are determined by two or more separate structural gene loci, each of which determines the amino acid sequence of a distinctive polypeptide chain. In such cases multiple isozymes are generated and the resulting heterogeneity is usually more complex than if just one locus is concerned. The polypeptide products of the different loci may separately form the various members of a set of isozymes or the different polypeptides may combine together, perhaps in different proportions, to form a rather more complex set of isozymes. Also there may be marked differences between the cells of different tissues in the quantities of the polypeptide products of the different loci. The relative amounts of isozymes determined by different gene loci may also show marked changes during the course of development, particularly during the fetal and neonatal periods.

Analysis and elucidation of such complex isozyme systems usually requires a combination of biochemical and genetic techniques although in some cases, for example, where the tissue distribution of the enzyme is so limited that it is not amenable to formal genetic analysis, one may have to rely almost exclusively on biochemical data. A useful practical point in these circumstances, however, is that the polypeptide

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products of alleles at the same locus are likely to differ by only single amino acid substitutions, whereas polypeptides coded at separate gene loci are likely to differ at quite a number of residues along the chain. Thus, in general, isozymes coded at the same locus tend to resemble one another in their biochemical and physical properties, whereas isozymes coded at separate loci tend to differ considerably in their properties.

Recent biochemical studies on the isozymes of human phosphoglucomutase (PGM) provide an interesting example of the way in which a biochemical approach can complement genetic methods of isozyme analysis. From conventional pedigree studies and population studies it was concluded that three distinct structural gene loci are concerned with the determination of human PGM and these loci were designated PGM_1, PGM_2, and PGM_3 (Hopkinson and Harris, 1969b). A series of electrophoretic variants attributable to the occurrence of multiple alleles at each of the three loci were identified, each allele resulting in the appearance of an altered set of isozymes characteristic of the particular locus. Secondary isozymes were also recognized (see section 1) but hybrid isozymes were not seen, suggesting that PGM is a monomer.

Studies of various biochemical characteristics—heat stability, molecular size, substrate specificity, and kinetic properties—showed that the PGM isozymes determined by alleles at any one locus were similar to each other but very different when compared with isozymes determined at the other PGM loci: for example, the PGM_2 locus isozymes are much more stable than the PGM_1 locus isozymes which in turn are more stable than the PGM_3 isozymes (McAlpine, Hopkinson, and Harris, 1970a). The PGM_2 isozymes are 15-20% larger than the PGM_1 and PGM_3 isozymes (Monn, 1969b; Santachiara and Modiano, 1969; McAlpine *et al*, 1970b). The PGM_2 isozymes are very active phosphoribomutases whereas PGM_1 and PGM_3 are very weak phosphoribomutases (Quick, Fisher, and Harris, 1972, 1974). Thus the biochemical data provide not only confirmation of the genetic hypothesis but also interesting new ideas on the possible functional significance of the PGM isozymes determined at different gene loci.

Conclusions

It is evident from this brief discussion that the multiple isozymes of any one enzyme can be generated in several different ways.

Secondary isozymes are ubiquitous but probably heterogeneous in origin and require special attention in genetic studies. In particular, it is essential to distinguish variation in secondary isozyme formation from genetic variation. However, it should also be appreciated that secondary isozymes may occasionally be helpful in detecting particular genetic variants. This was alluded to in the discussion on reactive sulphydryl groups in isozymes.

The occurrence of multiple isozymes due to allelic variation is also well established. A very large number of different enzyme loci have now been screened by electrophoresis and in many cases several different alleles, resulting in electrophoretically distinct isozymes of the same enzyme have been found (Harris and Hopkinson, 1972; Harris, Hopkinson, and Robson, 1974). A picture of extraordinary diversity in enzyme synthesis is emerging both in normal healthy people and in patients with pathological disorders. The functional significance of this individual diversity in enzyme constitution has been discussed earlier in this symposium by Professor Harris.

Multiple loci leading to the occurrence of multiple isozymes also appear to be quite common. Of about 60 complex isozyme systems analysed so far in human tissues, about a quarter are products of two or more separate loci. The different loci may give rise to separate sets of isozymes or the polypeptides may combine to give hybrid isozymes, although it should be noted that the failure to detect hybrid isozymes by electrophoresis does not preclude their existence *in vivo*.

Quite often the tissue distribution of isozymes attributable to different loci varies considerably and this presumably reflects different metabolic roles of these multiple forms of the same enzyme activity, though in certain cases it may reflect differences in the stability characteristics. Clearly it would be of interest to determine exactly how isozymes determined by different loci differ in their functional activities and also what processes are responsible for the particular distribution of isozymes which may be observed.

The occurrence of multiple gene loci determining the structures of a set of isozymes has a special clinical significance since mutant alleles at one locus may result in a marked or complete loss of activity in some isozymes but not in others. This may result in a clinical disorder, the characteristic features of which will depend on the normal distribution of the different isozymes in various tissues as well as on the particular isozymes affected and the degree of enzyme deficiency produced. The enzymes pyruvate kinase and aldolase are interesting examples of this phenomenon, since both are determined by more than one gene locus and in each case a pathological disorder has been recognized in which the products of just one locus and not the others have been affected by the presence of defective mutant alleles.

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