Current research on enzyme polymorphism

D.A. Hopkinson, MRC Human Biochemical Genetics Unit, London.

Introduction

Overall incidence of variation: Enzyme polymorphism is a well documented phenomenon in human populations. Estimates derived from electrophoretic studies indicate that approximately one third of all human enzymes exhibit genetic polymorphism where "polymorphism" is defined as the occurrence of heterozygotes with a frequency greater than 2%. Taken as a whole the data indicate that the average heterozygosity per locus is about 0.06 and this implies that any single individual in any human population is likely to be heterozygous at about 6% of the loci encoding enzyme proteins, for alleles which give rise to electrophoretically distinct isozyme forms (Harris & Hopkinson, 1976). The complexity of the isozyme patterns varies according to the subunit structure of the enzyme proteins and on average monomeric enzymes exhibit a higher incidence of genetic polymorphism than multimeric enzymes (Harris, Hopkinson & Edwards, 1977).

The data derived from electrophoretic studies of enzyme proteins can be used to obtain crude estimates of the incidence of mutation in the coding regions of the DNA of the structural genes for comparisons with the estimates derived from the direct molecular analysis of non coding (viz. the flanking and intervening) DNA sequences. Since the average enzyme polypeptide size is about 45,000 Daltons, corresponding to about 400 amino acid residues, it must be encoded by about 1,200 base pairs (bp) of DNA. About a third of all human enzymes exhibit polymorphism when examined by

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electrophoresis so that on average electrophoretic, i.e. charge-change, mutants occur with a frequency of about 1 in 3,600bp. Allowing for the fact that electrophoretic methods detect only about 30% of all the possible isozyme variants with single amino acid substitutions, the overall incidence of such polymorphisms in the DNA of enzyme polypeptide coding sequence must be in the region of about 1 in 1,000bp. This is an order of magnitude lower than the estimated incidence of variation in non coding DNA sequence (Jeffreys, 1979) demonstrable by direct analysis of restriction fragment length polymorphism (RFLP) using molecular probes.

Multiple loci: The occurrence of multiple gene loci encoding enzymes is a common phenomenon. Estimates based on the study of more than 100 human enzymes indicate that at least a quarter are encoded by more than one structural locus. These multiple loci, coding for similar though structurally distinct polypeptides have mostly arisen as a result of gene duplication during the course of evolution and the duplicate genes have subsequently diverged in structure as a result of point mutation. In many cases the polypeptides coded by each of the loci are synthesised together in the same cell but there are often marked disparities in the rates of synthesis in different tissues and at different stages of development. Such differences are important in forensic analysis since they may be used as indicators of the species and tissue of origin of an unknown biological stain or other types of sample and in some cases may also give an indication as to whether the specimen was derived from an adult or an infant.

Secondary isozymes: In addition to the complexities of enzymes due to allelic variation and mutliple loci there is also the phenomenon of secondary modification leading to the occurrence of multiple isozymes. This is a universal phenomenon and may arise due to a very great variety of causes. It can have a great nuisance value in forensic studies since secondary modification of enzyme proteins may mimic genuine genetic polymorphism and lead to misidentification. However some secondary changes of enzyme proteins are tissue specific and may therefore be useful in pointing to the origin of suspect human material in certain cases or they may provide clues as to the ways in which the material has been stored or handled prior to analysis.

Quantitative variation; "null alleles": A number of enzyme polymorphisms have been identified by quantitative assay techniques and in several cases these polymorphisms first came to light as a result of idiosyncratic responses to drug therapy. Their applications in the field of forensic science are limited however due to the difficulties of accurate identification of the variant phenotypes in the kinds of material usually available for analysis.

A more important category of quantitative variation is the so called "null" allele which is characterised by no discernable enzyme product. When such an allele occurs in a polymorphic enzyme system, such as PGM1, ACP1, ESD, difficulties may arise in paternity testing and of course individuals homozygous for such "null" alleles may present with specific clinical disorders, e.g. ADA, PEPD, GALT, αFUC. "Null" alleles have now been identified at almost every polymorphic enzyme locus and although in most cases their population

frequencies are low there are examples of very common "null" alleles at enzyme loci; for example the mitochondrial aldehyde dehydrogenase (ALDH2) polymorphism in Oriental populations (Goedde, Harada & Agarwal, 1979) and the recently identified glutathione-S-transferase (GST1) polymorphism in the European population (Board, 1981; Strange et al. 1984).

Recent developments

Enzyme purification: Protein purification has been revolutionised by the advent of affinity methods of separation and by the introduction of reverse phase high pressure liquid chromatography for the comparative analysis of amino acid sequences (see Scopes, 1982; Harris, 1985 for review). Immunoadsorption chromatography using polyclonal and monoclonal antibodies and affinity ligands based on substrates and inhibitors have been particuarly important. For example the synthesis of a specific affinity matrix for the purification of alcohol dehydrogenase (ADH) has recently led to the derivation of the entire amino acid sequence of different forms of ADH (Bühler et al. 1984) and the elucidation of the molecular basis of the human ADH2 polymorphism (Jornvall et al. 1984). Coincidentally, rapid progress has also been made in gene cloning experiments on human ADH (Duester et al. 1984; Ikuta et al. 1985) and it should now be possible to study the ADH polymorphisms using DNA prepared from white cells or any other nucleated cell rather than liver specimens, which were needed hitherto to examine the expressed gene product. This type of dual approach, which combines protein and DNA analysis, to investigate structure-function relationships in human enzymes is extremely powerful and while not

yet directly applicable in forensic science has already made important advances possible in the field of clinical medicine for the early and accurate diagnosis of specific genetic defects.

Enzyme separation techniques: The most powerful technique currently available for uncovering enzyme polymorphisms due to charge change substitutions is flat bed polyacrylamide gel isoelectric focussing. There are now many examples of subtypes, attributable to allelic variation, which are recognised by this method but are not visible by conventional methods such as starch gel electrophoresis. The IEF technique is capable of considerable experimental variation, using "spacer" molecules for example, to extend the range of separation and this procedure has infinite possibilities. The very exciting new method which depends on immobilised pH gradient is capable of even better resolution (Gianazza et al. 1983; Righetti et al. 1983), but has not yet been widely adopted for routine use.

Another high resolution technique is SDS - polyacrylamide gel electrophoresis and this procedure, which gives separations on the basis of molecular size is especially valuable for monitoring the phases of protein purification. Due to the denaturation of enzyme activity which occurs with the SDS method, it is not generally suitable for the detection of enzyme polymorphisms which depend on activity staining but this may be overcome by radio-labelling and immunological techniques. For example Waheed et al. (1983) recently identified a polymorphism of human arylsulphatase by SDS electrophoresis which appears to be due to an alteration in the number of carbohydrate side chains on the enzyme molecule. The probable site of the mutation is in the Asn-X-Thr(Ser) sequence

required for the attachment of asparagine-linked oligosaccharide side chains. Another mutant enzyme protein identified by SDS gel electrophoresis is a rare variant of hypoxanthine phosphoribosyl transferase with a neutral amino acid substitution which appears to have led to a change in the SDS-binding properties of the HPRT molecule (Wilson et al. 1983).

Enzyme detection methods: Many ingenious and specialised stains for enzymes have been devised and the principles underlying such methods which depend on the catalytic activity of the enzymes are given in Harris & Hopkinson (1976) and by Naylor (1980). The most important new and general alternative method to emerge in the past few years takes a different approach, and depends on the use of antibodies to detect the specific immunological determinants on the enzyme protein molecules. The enzyme under analysis is subjected to electrophoretic separation in agarose or polyacrylamide gels and the isozymes are then transferred electrophoretically from the gel onto a nitrocellulose filter. This electroblotting method, which was originally described by Towbin et al. (1979) for SDS gels also works well for non-denaturing polyacrylamide or isoelectric focussing gels. Passive transfer also may allow adequate retrieval of the enzyme protein (Gershoni & Palade, 1983).

The nitrocellulose blot is incubated with a specific antiserum raised against the purified enzyme protein (for example in a rabbit) and the enzyme-antibody complexes are detected with a second antibody (such as a goat anti-rabbit IgG) which carries some type of conjugated signal protein such as peroxidase or alkaline phosphatase which can be visualised by a conventional histochemical type

stain (e.g. Whitehouse & Putt, 1983; Blake et al. 1984).

The nitrocellulose filter provides a replica of the pattern which would be obtained by direct isozyme staining but the immunoblotting technique is usually considerably more sensitive than direct staining (Towbin & Gordon, 1984). The sensitivity depends largely on the quality of the first antibody and it is often possible to detect as little as a few nanograms of enzyme protein with a good antiserum. Even greater sensitivity may be achieved by the use of amplifying techniques such as "immunogold" (Moermans et al. 1984). So far the blot procedure has been used to analyse several human serum protein polymorphisms (e.g. Whitehouse & Putt, 1983; Whitehouse et al. 1985; Teige et al. 1985) but preliminary experiments with human enzymes (unpublished data) such as adenosine deaminase (ADA) and carbonic anhydrase (CA) gave convincing resolution of variant phenotypes in 2-5 µl aliquots of blood samples diluted down to 1 in 50. Thus the procedure may be expected to have a wide application to the study of human enzyme polymorphisms in forensic material.

Cloned human enzyme loci: The rapid progress which has been made in the cloning of human DNA sequences and their use as probes in clinical genetics and in linkage analysis has been referred to earlier and the specific applications of DNA probes in forensic science and paternity testing will be the subject of other presentations at this Conference. However in the context of this brief review of current work on human enzyme polymorphisms it is relevant to note that cDNA probes have now been generated for about half of the polymorphic enzyme loci listed by Harris & Hopkinson in

their Handbook of 1976-78 (see Beaudet 1985 for Bibliography of cloned human DNAs). Such probes make it possible to study in blood samples those loci where the enzyme is normally only expressed in less easily available tissue such as liver, gut and muscle. Also, using appropriate restriction enzymes it is often possible to reveal RFLPs at loci which are relatively invariant at the protein level.

Conclusions

1. The basic ideas promulgated more than 10 years ago about the incidence of human enzyme polymorphisms and their role in forensic science remain intact.

2. New enzyme polymorphisms can be identified by the application of the original methods; progress depends on the ingenuity of the investigator and the availability of substrates and other reagents necessary to generate specific staining methods.

3. The use of specific antibodies to detect genetic variation in human enzymes and other proteins in immunoblots prepared from IEF or conventional electrophoresis gels offers a powerful new approach for the forensic scientist. These procedures should be of general application but may be especially valuable for the detection and individualisation of trace amounts of human material on stains and other scene of crime objects.

4. The immunoblot approach will never be as powerful as DNA analysis for the identification of individuals in cases such as paternity disputes where conventional fresh blood samples are available, but it may have some advantages for the assessment of forensic material, especially that which has had to endure unfavourable conditions and where there has been some deterioration of the protein and DNA markers.

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