VARIANT POLYPEPTIDES IN HAIR

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TABLE OF CONTENTS

ABSTRACT

The purpose of this project was to develop techniques to characterize the polypeptides of hair proteins and to use these in identification of specific hair samples. The samples studied were those of normal individuals. The hair was solubilized in alkaline buffers containing 6 M urea and a reducing agent. Following alkylation of the proteins to the S-carboxymethyl derivative, polyacrylamide disc electrophoresis was done. Electrophoresis in a 7½% acrylamide gel showed a variant pattern of the α polypeptides in about 5% of caucasian samples and in 1 - 2% of black ones. Using 17½% acrylamide gels several variant patterns were found in the matrix proteins, the principal one occurring with a frequency of about 5%.

SUMMARY

This project followed the demonstration in our laboratory of an electrophoretic variant pattern of the α polypeptides (i.e. fibrous components) of human hair which occurred in about 5% of the population (Baden et al., 1975). This variant pattern was found in hair from all body regions of an affected individual and was transmitted in an autosomal dominant fashion. The hair appeared to be normal in structure and amino acid composition and the explanation for the variation was a polymorphism of one of the polypeptides most likely as a result of a single amino acid substitution. The first two years of this proposal have been involved with attempting to identify more electrophoretic variants using polyacrylamide disc electrophoresis.

We first explored the conditions for electrophoresis and noted again that 6 M urea had to be used in the electrophoresis in order to effect separation of the S-carboxymethylated fibrous proteins. We examined the pH range 2 to 10 and found that our modification of Davis technique worked the best (Baden et al., 1975). We experimented with electrophoresis at low pH using aminoethylated (Raftery and Cole, 1966) derivatives but this was not successful. The derivative prepared with iodoacetamide (Cavallito and Haskell, 1945) instead of iodoacetic acid also did not give satisfactory resolution. Using our original technique we found the incidence of the variant pattern in an additional group of caucasians of 4% to 5%. We did not find the variant in a sample of orientals (75 Japanese) or 25 black

Africans. In American blacks (100 specimens) the variant was found in two individuals which is close to the expected incidence of caucasian genes in that population.

We have found that our standard electrophoresis procedure is adequate for examining small specimens of hair. We can obtain excellent patterns using an 8 cm length of hair which weighs under 0.5 mg. The reaction is done in 5 ml of buffer and is either concentrated on Amicon membranes or dialyzed, lyophilized and brought up into a small volume (<0.1 ml).

Examination of the matrix proteins by polyacrylamide electrophoresis required the development of a new technology since these polypeptides ran as a single component by standard techniques. We employed the same buffers but used 17½% acrylamide which is highly cross-linked (the specific details of the procedures have been sent earlier to the LEAA) (Baden, 1976). Using this technique we have established the appearance of the usual pattern and have observed 5 variant patterns. One of these has been observed with a frequency of about 5%, the others at the 1% level. The patterns were entirely reproducible using multiple samples from the same individual. In the two instances where a family study was possible, a variant pattern could be identified in one parent and several of the children.

Having pursued the available techniques to their fullest capacity we began to undertake the separation of non-derivatized fibrous and matrix proteins for purposes of developing

antibodies to the separated fibrous and matrix components. The hair proteins were solubilized in 0.2 M Tris, pH 10.0, in 6 M urea with 0.1 M dithiothreitol (DTT) and then passed down a 200 cm Sephadex G 200 column using a buffer containing 0.01 M Tris, pH 8.3, 0.1 M sodium chloride, 6 M urea and 0.1 M DTT. Small aliquots of the separated proteins were alkylated and studied by disc electrophoresis to follow the separation. By repeated column runs it has been possible to separate the fibrous and matrix proteins. Aliquots of the pure components have been injected into rabbits and we have just recently observed antibodies to the fibrous components in two immunized rabbits.

INTRODUCTION

Although the appearance of an individual may be highly specific, the shape of individual features is not. With the exception of dermatoglyphics, it is unlikely that isolated structural features can be used with confidence in identifying individuals. This is because multiple genes are involved in determining morphological appearance and this is far removed from the primary gene product. On the other hand, when one can deal with primary gene products (i.e. structural proteins or enzymes) considerably more specificity is possible. Examples of this are the use of tissue types and blood types in identification.

The analysis of hair is limited by these same considerations. Although there are important differences between ethnic and racial groups, considerable similarities can be observed between individuals (Hrdy, 1973). Furthermore, considerably heterogeneity exists with respect to most morphological features of hair in a single individual. Because of the importance of hair in forensic medicine, it would be of considerable help to be able to deal directly with gene products in identifying hair. This project is directed to that aim.

From the work of Harris (1966, 1970) and others (Lehmann and Carrell, 1969), it has become apparent that variation exists in normal structural proteins and enzymes. An understanding of the basis of this variation evolved from the clarification of the coding of genetic information in the DNA of genes.

It was shown that the content and sequence of purine and pyrimidine bases in DNA provided the information which allowed the cell to synthesize proteins with a characteristic and reproducible amino acid sequence (Crick, 1967). In fact, a triplet of such bases was the code for a specific amino acid. This process of translation was mediated by an RNA molecule which was the actual template on which synthesis occurs. If an alteration occurs in a single triplet, such as a mutation, a different amino acid may be coded and the protein synthesized will now be different in amino acid composition. This may not give rise to a measurable change in physicochemical properties or functions.

When at the same genetic locus there exists a number of these modified genes, they are called alleles. Any individual cannot have more than two (from two paired chromosomes) at one locus, but the particular alleles can be any of those that might exist for the gene. Thus, if in the usual situation a person has AA, but B, C, D, E, etc., alleles exist in the population, one might find the combinations AB, BC, BE, etc. among various members of the population.

Recognition of the products of the alleles is not possible in all cases, but when one is dealing with a gene controlling a structural protein demonstration of the altered product is more feasible. The simplest method utilizes electrophoresis which depends on a change in charge of the protein as a result of the difference between the charge of the amino acid which has been substituted and the usual one. A number of examples have

been described and it is in part the basis for isoenzymes (e.g. enzymes with similar function, but different electrophoretic mobility) (Harris, 1969). Similar examples are found in structural proteins such as hemoglobin (Lehmann & Carrell, 1969). Of course, if the substitution has significant effect on the structure of the molecule, loss of function may occur and result in disease (Carson et al., 1956). We are here, however, primarily concerned with changes which only produce subtle alterations and function remains normal. When the incidence of an altered gene (and gene product) is 1% or greater, the term polymorphism has been used. The true extent of allelism is probably 3 times that observed by electrophoresis because many other subsitutions cannot be detected because no change in charge results.

In the case of keratinized tissues, variants of normal structural proteins in man were not known, although it had been shown by ourselves (Hrdy and Baden, 1973) and others (Schechter et al., 1969), that important variations could be detected between different animals. Even among the various primates, significant difference in the electrophoretic patterns of the solubilized hair proteins could be demonstrated. Our recent discovery (Baden and Lee, 1974) of a polymorphism of one of the α polypeptides of human hair and nail, however, has demonstrated that variants do exist in keratinized tissue. The failure to demonstrate these previously probably resulted from too little research in this area.

The bulk of hair is made of the cortex, whose cells contain two major structural proteins (Crewther, et al., 1965). These are the α-helical fibrous proteins (represented by filaments at a structural level) and the matrix ones (Fraser et al., 1972). They are present in about equal amounts. The filaments contain about 6 half cystine residues/100 residues, while the matrix proteins have about 30 half cystines/100 residues. It is thought that the matrix proteins are highly cross-linked by cystine and a few cross-links connect them to the α proteins, which are thus stabilized. The proteins are extracted in buffers at alkaline pH with urea and a reducing agent. By blocking the reduced cystines, the proteins are rendered soluble. Electrophoresis at pH 8.3 indicates the α protein contains 2 major polypeptides and several minor ones. At this pH all the matrix proteins migrate as one component. Research on wool proteins, however, indicates that there is considerable heterogeneity of the matrix (Frater, 1969).

In the variant pattern (Fig. 1) α_1 and α_2 are equal in intensity and half the intensity of α_2 in the normal pattern. The variant α_1 component presumably results from a polymorphism at the α_2 locus. In the variant hair pattern the normal α_1 is obscured by the variant α_1. This pattern is found in hair from all body areas of an individual and in nails. This alteration is transmitted in a codominant fashion in families.

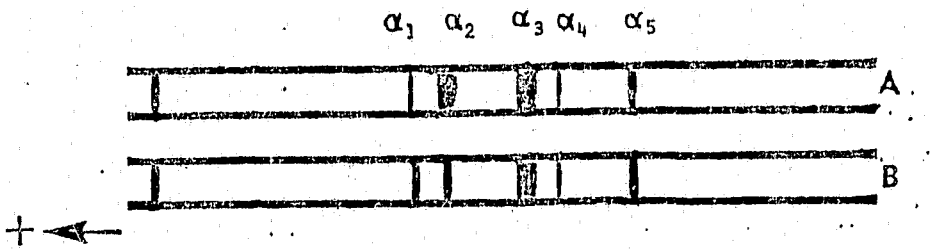

Figure 1 : Schematic drawing of disc electrophoretic patterns of hair SCM proteins. A is normal hair, B the variant hair. α refers to α-fibrous polypeptides.

	Ratio α_2/α_1
A	20
B	1

The importance of hair in forensic medicine cannot be over stressed. Present methods of analysis include morphological examination, which suffers seriously from lack of specificity (Noback, 1951). Chemical analyses have been directed toward measurement of metals which probably accumulate from contamination with environment (Yurachek, et al., 1969). Some trace metals could be endogenous metabolites deposited in the hair. However, specificity is again lacking, since content will depend on dietary intake or external exposure and cannot in many instances be considered entirely specific for an individual. Considerable overlap between people is bound to be found. Amino acid analysis with the exception of certain rare hereditable diseases (Pollitt and Stonier, 1971) is not exact enough because of the heterogeneous composition of the tissue and the overall close similarity between different individuals.

Variant polypeptides can be used as highly specific genetic markers, such as blood and tissue types. These would not be modified by exposure to environmental factors as might metal

content, etc. They are stable to weather and time and are not subject to manipulation. They are as stable as the hair itself. The value of such analyses, of course, will depend on the number of variant polypeptides that can be discovered. The history of this field in the past has indicated that once a polymorphism has been noted, additional ones are soon found. This is certainly related to the development of research efforts which previously had not been applied. We have only begun to look at this problem in the α-fibrous proteins of hair and a whole class of other structural proteins, the matrix ones, need to be looked at. Work on wool indicates that this class of proteins is more heterogeneous than the α ones and it is even more likely that numerous variants will be found.

TEXT

Extraction of Proteins

Hair samples were washed with petroleum ether and stored dry. The hair was extracted in 0.2 M Tris buffer, pH 10.0, containing 6 M urea and 0.2 M mercaptoethanol under nitrogen for 3 hours at 50° C. The supernatant was obtained by centrifugation and alkylation to give the s-carboxymethyl derivative was done at pH 9.0 with purified iodoacetic acid using 1.0 gm of acid for each 10 ml of solution. After 15 min excess mercaptoethanol was added and the sample dialyzed first against distilled water and then against the electrophoresis buffer. In some cases

iodoacetamide was used instead of iodoacetic acid and in others the aminoethylated derivative was prepared.

Electrophoresis in standard 7½% gels

We investigated the conditions for the best separation of the s-carboxymethyl polypeptides by doing electrophoresis in the pH range 2 - 10 using a number of buffer systems. The best separation was achieved with our modification of the Davis method (Baden, et al., 1975) which incorporated 6 M urea into the gel and 3 M urea into the buffer. Electrophoresis with derivatives prepared with iodoacetamide (Cavallito and Haskell, 1945) did not give as good results as those prepared with iodoacetic acid. Electrophoresis at pH 2.5 with the aminoethylated (Raftery and Cole, 1966) derivative also did not give good separation. Using our modification of the Davis method we found the incidence of the variant α polypeptide pattern in about 4% to 5% of an additional group of caucasians. We did not find the variant in a group of 75 Japanese or 25 black Africans. In American blacks the incidence was about 1% - 2% which is the expected incidence of caucasian genes in that population.

We have tried to apply our techniques to single strands of hair and used 3 cm lengths which weighed under 0.5 mg. The reaction was done in a volume of 5.0 ml and after dialysis the sample was concentrated on Amicon filters to about 0.1 ml. In some cases the samples were lyophilized and brought up to that

volume. Electrophoresis of 0.02 ml to 0.05 ml samples gave reliable patterns which could be readily visualized. We observed that 3 single samples from the same individual gave identical patterns both when an individual with the usual pattern and the variant pattern were tested.

Electrophoresis in 17½% gels

Examination of the matrix proteins by polyacrylamide disc electrophoresis required the development of a new technology since these polypeptides ran as a single component by standard techniques.

Polyacrylamide disc electrophoresis was done with the standard tube technique using the reagents described below. Certain precautions must be followed. The gel tubes must be thoroughly cleaned by soaking in nitric acid overnight and rinsed with dilute Photoflo solution (4 ml/500 ml) to allow removal of the gels. The running gel is allowed to polymerize overnight, while the stacking gel can be prepared in the usual manner. The electrophoresis is done with 2 milliamp/tube for about 3½ hr or 15 min after the marker dye runs off the gel. The gels were stained with Coomasie brilliant blue and scanned with a Canaldo densitometer.

Running gel

5.76 gm urea

2.0 ml sln A

2.8 gm acrylamide

147.2 mg N, N'-Methylene-bis acrylamide (Bis)

Brought to 16 ml, adjusted to pH 8.9 and 14 mg ammonium persulfate added.

Stacking gel

2.88 gm urea

1 ml sln B

2 ml sln C

1 ml sln D

Brought to 8 ml and pH 6.7

Solution A

48 ml 1 N hydrochloric acid

36.6 gm Tris

.025 ml N, N, N', N'-Tetramethylenediamine (Temed)

Brought to 100 ml and pH 8.9

Solution B

48 ml 1 N hydrochloric acid

5.98 gm Tris

.46 ml Temed

Brought to 100 ml and pH 6.7.

Solution C

10 gm acrylamide

2.5 gm Bis

Brought to 100 ml

Solution D

4 mg riboflavin

100 ml H_2O

The running buffer contained 3 M urea, 0.02 M Tris and 0.005 M glycine adjusted to pH 8.3 with hydrochloric acid.

Using this technique we have established the usual pattern (Fig. 2) of the matrix proteins which consists of four bands.

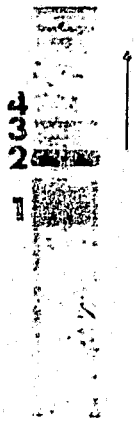

Figure 2

Bands 1, 2 and 3 appeared to consist of two very closely migrating components which varied in their degree of separation in different runs. A number of variants were observed and these were verified by analyses in samples from the same individual. The most frequent variant was an extra band between bands 2 and 3 which occurred with an incidence of about 5%. In two family studied this variant appeared to be transmitted in an autosomal dominant fashion. Other variants that were observed in less than 1% of samples included an extra band between 1 and 2, an absence of band 4, an absence of band 3 and 4 and an unusual position of band 4. All these patterns were repeated in triplicate and gave the same results.

Antibody studies

Having pursued these electrophoretic techniques to their full capacity we began to undertake the separation of the non-

derivatized fibrous and matrix proteins for purposes of developing antibodies to the separated fibrous and matrix components. It was felt necessary to use unmodified proteins since considerable cross-reactivity is induced by making the s-carboxymethyl derivative. The hair proteins were solubilized in 0.2 M Tris, pH 10.0, in 6 M urea with 0.1 M dithiothreitol and then passed down a 200 cm Sephadex G 200 column using a buffer containing 0.01 M Tris, pH 8.3, 0.1 M sodium chloride, 6 M urea and 0.1 M dithiothreotol. Small aliquots of the separated proteins were alkylated and studied by electrophoresis to follow the separation. By repeated column runs of selected fractions it has been possible to separate the fibrous and matrix proteins. Aliquots of the pure components have been injected into rabbits and we have obtained antibodies to the fibrous components. We have not as yet rated antibodies to the matrix components. The plan is to prepare antibodies to proteins purified from different individuals and compare the reaction of the different antigens to different antibodies to determine if antigens peculiar to different individuals can be detected. If different antigens could be detected these could be used for identification in a very specific way.

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