DIFFERENCES IN THE PRIMARY STRUCTURE BETWEEN ISOENZYMES OF HORSE LIVER ALCOHOL DEHYDROGENASE.

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Summary. The main form of each of the three isoenzyme groups of horse liver alcohol dehydrogenase has been investigated by fingerprinting techniques. Three amino acid substitutions causing differences in the primary structure between the two protein chains forming the isoenzymes were detected. These replacements make one chain more hydrophobic than the other but give no explanation for the apparent differences in charge between the isoenzymes. All three substitutions are compatible with single base mutations and a common genetic origin is suggested.

Horse liver alcohol dehydrogenase is known to consist of isoenzymes. There are three groups. The main form of the most acidic group was the first known isoenzyme, crystallized in 1948 (Bonnichsen and Wassén 1948) and later called isoenzyme E (Theorell et al. 1966) or EE or 3 (Pietruszko et al. 1966). The main form of the second group was crystallized in 1966 (Theorell et al. 1966) and called ES or 1 (Pietruszko et al. 1966). The crystallization of the main form of the third and most basic group (Theorell 1968), called SS, has not yet been described. It has, however, been isolated by Dr. Å. Åkeson in this laboratory in electrophoretically pure form.

It has been shown that the EE isoenzyme is a dimer (Butler et al. 1969) and that it is composed of two probably identical protein chains (Jörnvall and Harris 1969). This chain is called the E-chain below.

The existence of a common subunit in the EE and ES isoenzymes is indicated by immunology (Pietruszko and Ringold 1968). From dissocia-

tion/reassociation studies it is known (Pietruszko et al. 1969) that the ES isoenzyme is composed of two non-identical subunits, one of which (E-chain) is electrophoretically identical with the subunit from the EE isoenzyme, and the other (called S-chain below) with the subunit from the SS form. The nature of the difference between the two subunits (E and S) of the ES isoenzyme was unknown since total amino acid analyses of EE and ES had not revealed any significant differences (Theorell et al. 1966).

In the present study, therefore, the ES isoenzyme was carboxymethylated with ^{14}C and then digested with trypsin. The tryptic digest was "fingerprinted" by multidimensional electrophoreses at pH 1.9, 3.5 and 6.5 and by chromatography (for experimental methods see Jörnvall and Harris 1969). These fingerprints were compared with a similar digest from EE isoenzyme. It was then found that the ES digest had all the peptides occuring in the EE digest. This again shows that the E-chain of the ES enzyme is identical with the E-chain of the EE enzyme. Furthermore most of the peptides in the ES digest were found in the same amount as in the EE digest, based on the strength of the ninhydrin colour and of the radioactivity. This clearly establishes that the S-chain mainly gives the same peptides as the E-chain, in other words the two chains are very similar.

There were, however, a few differences between the digests. Four of the peptides in the ES digest were found in smaller (about half) amounts than the same peptides in the EE digest. In addition, four peptides, never detected in EE digests, were found with a yield of about half in the ES digest.

The amino acid sequences of the four extra peptides were established. It was then found that the extra peptides had sequences homologous to one each of the four "ordinary" peptides which were found in reduced amounts in the ES digest. In each case there was complete iden-

tity except for one single residue which had been replaced by another amino acid. Furthermore two of these four peptides overlapped (caused by a partial split by trypsin). Therefore the differences found constitute only three unique replacements, which are shown in the table.

These results clearly show that the S-chain is identical to the E-chain except at a few places, where an exchange in a single residue has taken place. Thus, in the digest of the ES enzyme the corresponding stretches are found in their two different forms, each in a recovery of about half from the form occurring in the digest of the EE enzyme.

Knowing this, highly purified SS isoenzyme was also investigated. A tryptic digest of the carboxymethylated form was compared with the ES and EE enzymes in the same way as above. It was then found that the SS isoenzyme had the "extra" peptides from the S-chain in full recovery and that the "ordinary" peptides from the E-chain were completely lacking. No other differences from the digests of either the EE or ES forms were detected. This clearly establishes that the S-chain from the ES isoenzyme is indeed identical with the subunit from the SS enzyme and that the latter enzyme is composed of two such chains.

It is then suggested that the E- and the S-chains have a common genetic origin and that they have evolved into two different chains by a few mutations. Noticably, each of the three exchanges found are mutations that require only one base change in the genetic codon (Nirenberg et al. 1965). From the three possible dimeric combinations of the two chains the main form within each isoenzyme group is obtained.

The work on the complete sequence of the EE isoenzyme has advanced so far that the positions of the three substitutions along the protein chain may, at least preliminarily, be indicated. Thus replacement number 1 is close to the N-terminus, the exact number being amino acid 17. Replacements number 2 and 3 are close together, separated only by 6 other residues. The second substitution is with some uncertainty situated at

about number 130 from the N-terminus and the third in such case at number 137.

It is known that the three isoenzymes differ in substrate specificity. EE is active towards ethanol but not towards 3β-OH-5β-steroid alcohols, SS has less ethanol activity but considerable steroid activity and ES has activities in between the other two forms (Theorell 1968). This must obviously be explaned by differences in the substrate binding sites of the two types of chains in the native enzymes.

It is then of great interest to notice that in each of the three mutations a residue in the E-chain is replaced by a more hydrophobic one in the S-chain. Alterations in the three-dimensional structure of the native enzymes caused by these replacements might be an explanation of the different substrate specificities. Any such alterations could not, however, be very extensive, as one subunit, without change of its properties, can fit into a dimer with either type of chain (Theorell 1968). It would then be tempting to suppose that the mutations might play a more direct role in the substrate binding site, especially as the steroid substrate is also more hydrophobic than ethanol. If so, the region of mutation number 1, the region of mutations 2 + 3 and a third region containing the cysteine residue already known to form part of the active site (Harris 1964; Lie and Vallee 1964) might be close together in the three-dimensional structure. The hydrophobic changes could also be of interest in regard to the increased solubility of SS, versus EE, in ethanol (Åkeson 1968) and other changes in solubility in different solvents.

The isoenzymes EE, ES and SS are clearly distinguished by zone electrophoresis at different pH's. The apparent isoelectric points, derived from pH of zero mobility versus starch are approximately 8.5, 9.4 and 9.8 respectively in TRIS and carbonate buffers, 0.1 M, on polyacrylamide gels (Jörnvall 1966). It is then of interest that the three mutations found give no explanation of the more basic nature of the S-chain.

Table

Amino acid exchanges between the two types of chains.

Mutation number	Position along the protein chain	E-chain	S-chain
1	17	glu	gln
2	130 (?)	thr	ile
3	130 (?) + 7	arg	ser

Of course, other mutations, not yet found, may also exist. It seems, however, not improbable that all replacements changing the charges of peptides were detected. It may be noted that the second mutation was found although it represents only an exchange of two neutral residues. The present finding might therefore indicate that the more basic nature of the S-chain is due to conformational changes or other factors and not to a relative increase in basic amino acid residues. It should, however, be noticed that the two chains are distinguished by electrophoresis in 8 M urea as well (Pietruszko et al. 1969), where the peptide chains ought to be unfolded.

The work on the structure of all three groups of isoenzymes is being continued. At present more than 350 (over 90 %) of the amino acids of the E-chain have been found in peptides and a provisional sequence of the whole chain established.

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