IDENTIFICATION OF A REACTIVE ARGINYL RESIDUE IN HORSE LIVER ALCOHOL DEHYDROGENASE

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Summary: One arginyl residue per subunit of horse liver alcohol dehydrogenase modified with [^{14}C] phenylglyoxal has been identified as Arg-84. A single radioactive peptide isolated from a chymotryptic digest of the labeled enzyme consists of the sequence Val-83 to Leu-92. This finding is consistent with those of previous chemical modifications and with the three-dimensional structure of the protein indicating that Arg-84 is in an unusual environment. Another radioactive fraction of the digest contains a non-peptide substance tentatively identified as phenylglycine that presumably derives from a labile derivative of a second arginine residue. The lability of this second arginine derivative may relate to its probable location at the active site of the enzyme.

Introduction: The specific arginyl reagents 2,3 butanedione and phenylglyoxal inactivate alcohol dehydrogenase from horse liver (1). Loss of activity correlates with modification of two arginyl residues per subunit and has been shown to be due to abolition of coenzyme binding. Arginine modification also markedly reduces the rate of carboxymethylation of Cys-46 by iodacetate (2) leading to the conclusion that inactivation of alcohol dehydrogenase is due to modification of the adjacent residue, Arg-47. These results are entirely in accord with those of x-ray structure analysis which indicate that Arg-47 interacts with the pyrophosphate bridge of bound coenzyme (3). Enzyme labeled with [^{14}C] phenyglyoxal has now been examined in order to get direct evidence for the arginyl residue(s) modified. One of these has been identified as Arg-84. These results are analogous to those obtained with other chemical modifications of alcohol dehydrogenase using different reagents (4-5). Arg-84 is thought to be in a reactive region of the protein but is not known to be important for catalytic activity.

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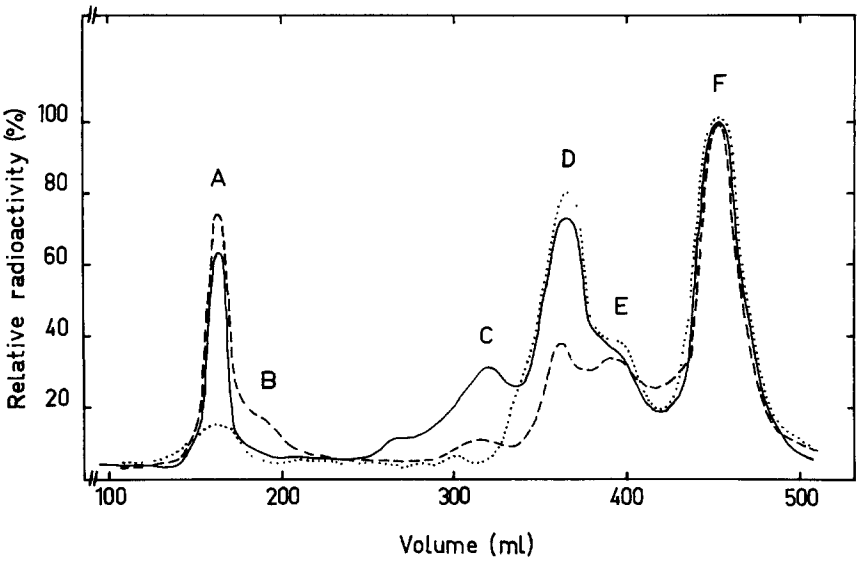

Figure 1: Exclusion chromatography on Sephadex G-50 (2.5 x 100cm) in 0.1 M ammonium bicarbonate of a chymotryptic digest of ^{14}C phenylglyoxal-treated horse liver alcohol dehydrogenase. Three different experiments are marked ——, --- and ····

MATERIALS AND METHODS: Horse liver alcohol dehydrogenase (Boehringer-Mannheim Corp.) contained mainly the ethanol-active isozyme. Labeling was carried out with [^{14}C] phenylglyoxal in the absence of coenzyme as described previously (1,2). Approximately 1.6 arginyl residues per subunit were modified and activity decreased to 20% of the unmodified control. The labeled protein was gel filtered through Bio-gel P-4 equilibrated with 125 mM NH_4HCO_3, pH 7.9, to remove excess reagent and then lyophilized. A portion of the resultant white powder was redissolved in and dialyzed against bicarbonate buffer. Approximately 13% of the radioactivity was lost by this treatment. The rest of the protein was digested with TLCK-chymotrypsin (6) and the peptide mixture was separated by chromatography on Sephadex G-50 fine in 100 mM NH_4HCO_3. The radioactive fractions were pooled and purified further on paper by high-voltage electrophoresis in different buffers and by chromatography in n-butanol: acetic acid; water; pyridine (15: 3:12:10) as previously described (6). Pure products were eluted with water and analyzed for composition, N-terminal residue and amino acid sequence (6).

RESULTS: Three different preparations were examined. The exclusion chromatography elution profiles of chymotryptic peptides are shown in Figure 1. Each profile consists of three major radioactive peaks (A,D and F in Figure 1) apart from some that are minor or found inconsistently (B,C,E). The labeled products corresponding to the major fractions were analyzed further.

The first radioactive peak (A in Figure 1) eluted from the Sephadex

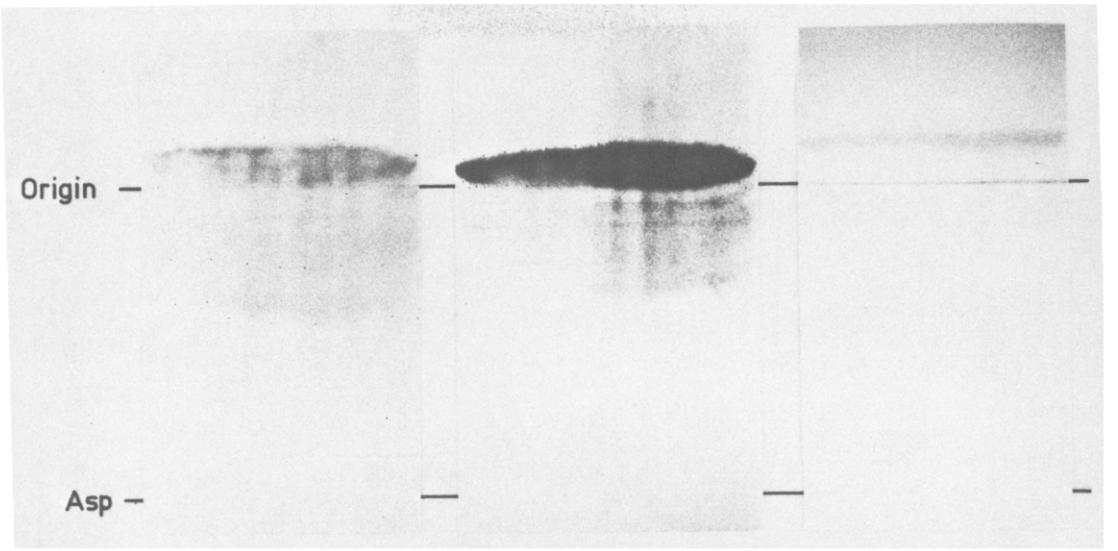

Figure 2: Autoradiographs obtained after electrophoretic separations on paper at pH 6.5 of the modified peptide, corresponding to the pooled fraction D in Figure 1. The separate parts are derived from each of the three labelling experiments. Anode at bottom.

column close to the void volume, was the least ^{14}C labeled product of the major fractions, and in one preparation was virtually absent. Most of this fraction precipitated upon electrophoresis at pH 6.5. It seemed to correspond to large peptide fragments resulting from incomplete digestion, as observed previously with different chemical modifications (7,8). All properties of this peak are consistent with this interpretation.

The second major radioactive peak (D in Fig. 1) contained about 40% of the total ^{14}C-content in the best digested samples, and corresponded to a specifically labeled product as shown in Figure 2. It was purified by electrophoresis at pH 6.5 and 1.9 and by paper chromatography. Amino acid analysis indicated that it corresponded to the sequence from Val-83 to Leu-92 (6). Based on the specificity of phenylglyoxal (1) Arg-84 was thought to be the residue labeled. This conclusion was verified by Edman degradations. Radioactivity appeared predominantly in the extract removed after the second degradative step corresponding to the arginyl residue at position 84 (6).

The third major radioactive peak (F in Figure 1), containing about 50% of the total ^{14}C-content, also yielded one labeled product on further purification. Amino acid analysis of this material with or without previous acid hydrolysis revealed only one compound which eluted from the analyzer in a position very close to phenylalanine. No usual N-terminal amino acid was detectable and attempts at Edman degradations were unsuccessful. Instead, dansylation revealed a derivative with properties similar but not identical to those of dansyl-phenylalanine in all chromatographic systems (6). The relative differences in mobilities between the dansyl product and dansyl-phenylalanine in the solvent systems were comparable to those between dansyl-glycine and dansyl-alanine. Autoradiography of the chromatogram showed this product to be ^{14}C-labeled. The same dansyl derivative was obtained both with and without prior acid hydrolysis. It would appear, therefore, that the radioactive product in the third Sephadex fraction is not a peptide but a ^{14}C-labeled free amino acid related to phenylglyoxal, the initial source of radioactivity, with properties similar to phenylalanine. It is probable that this substance may be phenylglycine and that it arises from either transamination of the reagent (9, 10) or from disproportionation of a modified residue subsequent to labelling.

DISCUSSION: These results concern both the identification of modified arginyl residues in proteins and the relationship between protein structure and reactivity of amino acid residues. Of the 12 arginyl residues per subunit of horse liver alcohol dehydrogenase only two are modified selectively by either butanedione or phenylglyoxal (1). One of these is now shown to be Arg-84. Several lines of evidence suggest that the other is Arg-47 (2) at the active site, even though the corresponding peptide has not been isolated directly. The involvement of the latter residue is consistent with x-ray crystallography (3), loss of enzymatic activity accompanying arginine modification (1), and influences on specific carboxymethylation of the adjacent Cys-46 in the butanedione-modified enzyme (2).

The failure to isolate a labeled peptide from the region around Arg-47 might be due to the instability of phenylglyoxal-modified arginyl residues. This complication is a limitation in the identification of such residues in proteins (9). Unless the conditions employed are particularly favorable, labeled peptides will not survive fragmentation and isolation procedures. Moreover, phenylglyoxal adducts might be expected to be quite sensitive to the presence of a metal ion or cysteine as at the active site of liver alcohol dehydrogenase. Lability of modified active center arginyl residues has been observed previously (11) suggesting that features contributing the unusual reactivity of such residues towards chemical reagents may also affect the stability of the reaction product.

Presently there is no information to indicate that Arg-84 is directly involved in the catalytic mechanism of horse liver alcohol dehydrogenase. The x-ray structure shows that it does not interact with either substrate or coenzyme, though addition of coenzyme protects the native enzyme against modification. The present identification of this residue in three different experiments precludes an artifact, and the selective modification of Arg-84 suggests that it is located in a region of the protein that is particularly reactive. It is exposed on a surface on the opposite side of the catalytic domain in relation to the entrance to the active site region, and far from known effects of protein structure or subunit interactions (3). Interestingly, two adjacent lysine residues, Lys-325 and Lys-159, are also unusually reactive, Lys-159 toward reductive methylation (4), and both toward a chlorinated coenzyme derivative (5). Together, the lysyl residues and Arg-84 define a region of enhanced reactivity or accessibility. The possible function of this area is unknown but the results exemplify the importance of local environment in determining unusual chemical reactivities of amino acid residues in proteins. Active center residues of enzymes frequently exhibit such reactivity, but apparently it may also be pro-

duced in other regions as shown in the present case, and perhaps in other instances where unexpected modifications are observed as well (12).

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