

Short Communications

On the Determination of the N-Terminal Groups of Horse Liver Alcohol Dehydrogenase

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In connection with studies on the structure of horse liver alcohol dehydrogenase (LADH) going on in this laboratory an attempt was made to determine the amino end-groups using the 1-fluoro-2,4-dinitrobenzene (FDNB) method. The analyses were performed on native as well as heat-denatured and chemically modified enzyme.

Seven different preparations of LADH were analysed under various conditions (Table 1). The enzyme was prepared essentially according to Dalziel.¹ In experiment 6, however, the crystallization of the enzyme was performed exclusively with ammonium sulphate and in experiments 2 and 5 the main component¹ of LADH was isolated and used for the analyses. In some experiments the enzyme was oxidized² or carboxymethylated.³ Condensation with FDNB was performed according to Levy *et al.*⁴ in experiments 1-3 and according to Sanger⁵ in 4-7. The DNP-protein was hydrolyzed and the hydrolysate fractionated as described by Biserte *et al.*⁶ All phases were chromatographed on Whatman No. 1 paper according to Phillips⁷ and in some experiments analysed according to Spackman *et al.*⁸ after hydrolysis with ammonia.⁹ The DNP-amino acids were determined by measuring the light absorption at 360 m μ ¹⁰ after elution from the paper.⁶ The values reported have not been corrected for losses.

The analytical results are shown in Table 1. Several DNP-amino acids were found but in widely varying amounts and with a mole ratio amino acid/protein always below one,

except in the case of arginine. Furthermore, the ether soluble DNP-amino acids were not always the same and in some cases none at all were found. With 12 N HCl hydrolysis for 4 h of some samples in experiment 1 neither DNP-proline nor DNP-glycine was found. The destruction products described by Scanes *et al.*¹¹ were not present. In experiments 2, 5, and 6, however, very small amounts of unknown ether soluble substances were found. No DNP-cysteic acid was detected in experiment 2 and no yellow spot corresponding to DNP-CM-cysteine in experiments 6 and 7. In no case was the fast moving brown substance derived from cystine and described by Bettelheim¹² found. Unless excessive destruction has occurred, as for example has been reported for DNP-serine,⁴ these experiments exclude as N-terminal all amino acids giving ether soluble DNP-derivatives.

Of the amino acids giving water soluble DNP-derivatives, arginine could not at first be ruled out as N-terminal. Owing to the large amount of ϵ -DNP-lysine present in the water phase the quantification of DNP-arginine was very difficult. To avoid this complication the water phases in experiments 3-7 were run through a column of talc^{13,6} equilibrated with 1 N HCl. This was expected to separate the free amino acids from the DNP-derivatives. The retarded DNP-amino acids were eluted^{13,14} and once more treated with FDNB^{4,5} in experiments 3-5. In all these experiments DNP-arginine was found, but in highly varying amounts. This could possibly be due to a partial masking of eventual N-terminal arginines, especially in view of the unusually high content of SH-groups¹⁵ in LADH. A modification of these SH-groups could be expected to unfold the structure and prevent possible crosslinking and thus make the N-terminal amino acids more accessible. In experiment 6, therefore, all SH-groups were carboxymethylated and using the above method^{13,6}, a surprisingly large amount of DNP-argine (2.84 moles/mole of enzyme) was found. There was, however, a background of other DNP-amino acids.

Table 1. Moles of DNP-amino acid found per mole of LADH.

Exp. Condensed enzyme	1 native	2** oxidized	3 90°C, 4 min	4*** native	5 native	6 CM-LADH	7 CM-LADH
DNP-asg	—	+	—	—	—	} 0.39	—
DNP-glu	—	+	—	—	—		—
DNP-thr	—	—	—	—	—	0.13	0.15
DNP-ser	—	+	—	—	—	0.16	0.11
DNP-gly	—	—	—	—	—	0.33	0.03
DNP-ala	—	+	—	—	—	0.50	—
DNP-val	—	+	—	—	—	0.89	—
DNP-ileu } DNP-leu }	—	+	—	—	—	0.54	—
DNP-phe	—	+	—	—	—	0.64	0.08
di-DNP-lys	—	+	—	—	0.08	0.47	0.07
di-DNP-his	—	—	—	—	—	0.15	—
DNP-arg	*	—	+	1.86	0.29	2.84	—

* A Sakaguchi-positive spot was detected but the R_F -values did not quite agree with those of DNP-arginine. The large amount of ϵ -DNP-lysine present could, however, conceivably cause a change in the R_F -values.

** Semi-quantitative; the amounts of detected acids being of the same order of magnitude as in experiment 6.

*** Traces of all other soluble amino acids were found by analysis according to Spackman *et al.*⁸ after hydrolysis with ammonia.⁹

One possible source of error now suggested itself; if free arginine, contrary to earlier statement,¹³ was retarded on the talc column, it could be eluted together with the DNP-amino acids and the subsequent analysis result in a false or at least too high value for DNP-arginine. This suspicion was further substantiated when the first eluate (1 N HCl) from the talc column in experiments 5–7 was analysed for free amino acids. Over 80 % of the original amounts of those amino acids which had not reacted with FDNB were recovered. The yield of arginine was, however, much lower, or 40–60 %. To test the reliability of the method a talc column was charged with about equal amounts of some free amino acids including arginine and eluted in the usual way with 1 N HCl. The elution was continued until more than twice the volume needed to elute the amino acid peak, as determined by the ninhydrin reaction, had passed the column. The elution was then continued with 80 % ethanol containing 0.3 % ammonia.¹⁴ The results are shown in Table 2, and it can be seen that arginine was considerably retarded on the talc column in 1 N HCl.

This effect must obviously depend on the volume of the first eluate, the size of the talc column and the concentration of arginine. Since, of all the amino acids, arginine seems to be selectively retarded, the original method¹³ using FDNB to condense the second eluate, seems unsuitable. Special precautions would be necessary for the determination of N-terminal arginine. The high and variable amount of DNP-arginine found in experiments 3–6 is thus explained. In experiment 2 no DNP-arginine was found since a talc column was

Table 2. Per cent recovery of known amounts of some free amino acids from a talc column.

Amino acid	Eluant 1	Eluant 2	Total
phe	92.8	—	92.8
lys	95.2	—	95.2
his	93.6	—	93.6
arg	71.1	24.3	95.4

not used and in experiment 7 the false DNP-arginine was avoided by using methoxy-carbonyl chloride¹⁶ instead of FDNB for the second condensation. In the latter version the method appears quite safe. Thus, according to the present investigation, arginine also seems to be excluded as N-terminal amino acid.

An interesting point is that the background of DNP-amino acids was found in experiments 2, 6, and 7, *i.e.* in those cases where the SH-groups were modified. This could mean that the modifications had caused splitting of peptide bonds to a slight extent. The background could, however, also be due to amino acids or small peptides adsorbed to the enzyme. This material might then be inaccessible to FDNB, due to masking, unless the molecule is opened up by modifying the SH-groups. Oxidation² and carboxymethylation³ should not cause any splitting of peptide bonds. Since there are several hundred peptide bonds per molecule, even a very slight degree of splitting would, however, be sufficient to explain the detected small background of DNP-amino acids.

Adsorbed amino acids or small peptides have been found in yeast alcohol dehydrogenase¹⁷ which was crystallized using ammonium sulphate. It is interesting that when LADH was crystallized with ammonium sulphate as in experiment 6 the background was more dense than when ethanol was used in the otherwise identical preparation of the enzyme in experiment 7. The solvents used to remove excess FDNB before hydrolysis in experiments 6 and 7 were analysed for DNP-amino acids. These had to be applied to the chromatography paper in a very dilute form due to the large excess of FDNB, dinitrophenol and dinitrophenylamine. In experiment 6 only these compounds were found, but in experiment 7 three spots appearing as DNP-amino acids could be distinctly seen against a yellow background. Due to this background the quantification and identification of the spots were difficult, but the position of one spot agreed closely with DNP-serine (about 0.6 mole/mole of protein), the position of the second with di-DNP lysine (somewhat less) and that of the third with DNP-aspartic acid. Amino acids liberated by a splitting of peptide bonds during carboxymethylation would have been removed by the following dialysis and thus not found by condensation of the protein with FDNB. These findings therefore support the adsorption theory. In that case at least

some of the adsorbed material must be free amino acids as DNP-amino acids were found before hydrolysis. If adsorbed, the material must stick at least partly to the enzyme, even after rather drastic treatment. Before carboxymethylation the protein was thoroughly dialyzed against acid (pH 4) to displace the intrinsic zinc and after the reaction against alkaline solution to remove the reagents. If the background came from adsorbed material it is thus possible that still more originally existed but was removed in the dialyses.

The nature of the N-terminal groups is being studied further as well as the question of adsorbed amino acids or small peptides.

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