AMINO ACID SEQUENCE AROUND A REACTIVE CYSTEINE OF YEAST ALCOHOL DEHYDROGENASE.

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SUMMARY - The reaction of yeast alcohol dehydrogenase with iodoacetate produces a loss of 90% of the enzymatic activity when 2.2-2.4 equivalents of carboxymethyl groups are incorporated. After CNBr cleavage of the ^{14}C-carboxymethylated enzyme the more radioactive fragment was maleylated and digested with trypsin. A tryptic peptide, containing about 70% of initial radioactivity, was purified and sequenced. The cysteine found to be more reactive with iodoacetate in our experimental conditions is different from that found in previous works. It also appears to differ from the cysteine residues found to be reactive towards other thiol reagents.

Yeast alcohol dehydrogenase is a tetramer composed of four identical or highly similar polypeptide chains of approximate molecular weight 35,000 (1,2). Inactivation experiments (3,4,5) and studies on coenzyme binding (6) led to the proposition that each subunit contains a single coenzyme binding site and a single sulphydryl group necessary for enzymic activity. Auricchio and Bruni (7) showed that the "essential" -SH groups of yeast alcohol dehydrogenase do not serve the function of binding the coenzyme. More recently, however, the number of essential sulphydryl groups and coenzyme binding sites have been re-examined.

Following the reaction of the native enzyme with iodoacetate, Dickinson (8) concluded that only two thiol groups are essential for enzymatic activity. Similar findings were reported by Twu et al.(9),who also showed that buthyl isocyanate inactivate yeast alcohol dehydrogenase and the reaction is with two cysteine residues, each of which is in the same unique sequence of amino acids in the protein (10). The

inactivation of yeast alcohol dehydrogenase with iodoacetamide was found to involve four thiol groups per mole of enzyme (8,10). Two major radioactive peptides were obtained by Twu et al.(9) after reaction with (^{14}C)iodoacetamide, one corresponding to the butylcarbamoylated peptide and the other similar to that isolated by Harris (1).

In the present study we report that the inactivation of yeast alcohol dehydrogenase with iodoacetate involves a cysteine residue, which is different both from that found by Harris (1) and that found by Twu and Wold (10).

EXPERIMENTAL AND RESULTS

Reaction with Iodo(2-^{14}C)acetate - Yeast alcohol dehydrogenase (purchased from Boehringer, Mannhein), dissolved in 4 x 10^{-2}M phosphate buffer, pH 7.0, at a concentration of 15 mg/ml, was incubated with a 16 fold molar excess of iodo(2-^{14}C)acetate (The Radiochemical Centre, Amersham, U.K.). The reaction was carried out at 0°C. Samples were assayed at zero and later times both for enzymic activity and radioactivity. Enzyme activity was determined as described by Wallenfels and Sund (11). Radioactivity measurements were made on samples promptly mixed with acetone-hydrochloric acid (95:5,v/v). The samples were freed of excess reagent by washing the obtained precipitates with several changes of acetone-hydrochloric acid, centrifuged down and dried. The samples were redissolved in 98% formic acid and the radioactivity was assayed in a Tri-Carb liquid scintillation spectrometer (Packard).

The activity of the enzyme was 9.6-11.4 % after 2.5 hours of incubation. Under these conditions 2.2-2.4 moles of iodo(2-^{14}C)acetate were found per mole of yeast alcohol dehydrogenase, assuming a molecular weight of 140,000 for yeast alcohol dehydrogenase (1,12).

CNBr cleavage - To the 10% active enzyme was added dithiothreitol to react with the excess of radioactive iodoacetate and complete carboxymethylation was performed using an excess of non-radioactive iodoacetate; the cold iodoacetate was so much as to react with the excess of dithiothreitol thiol groups and to be in a two-fold excess over the protein sulphydryl groups, assuming a number of 36 sulphydryl groups per mole of enzyme (1).

The reaction was carried out in the presence of 0.33 M Tris, 8 M urea, at pH 8.5, under nitrogen for 3 hours at room temperature.

The excess reagents were removed by dialysis against several changes of HCl, pH 3, and the carboxymethylated yeast alcohol dehydrogenase was freeze-dried.

The freeze-dried carboxymethylated enzyme (150 mg) was dissolved in 7 ml of 70% formic acid and about 250 mg of cyanogen bromide were added. The reaction was allowed to proceed for 24 hours at room temperature. Following incubation, the sample was diluted 20-fold with distilled water and the excess reagents were removed by evaporation into a dry ice trap. The content was then evaporated to dryness twice from water by lyophilization.

Isolation and purification of radioactive fragment - The lyophilized material was separated in two fractions, by four consecutive extractions with 5 ml of pyridine-acetate buffer (10% pyridine, 0.5% acetic acid), pH 6.5. The radioactivity of the pooled soluble fraction (containing about 90 mg of protein) was found to be 28-31% of the initial radioactivity.

Both the soluble and insoluble fractions obtained from a small scale experiment were digested with trypsin by incubation at 37°C and pH 8.0 for 4 hours. The resulting digests were subjected to high voltage paper electrophoresis at pH 6.5. The radioactive peptides were localized by autoradiography. A qualitative scheme is shown in fig.1.

The insoluble fraction was dissolved in 4 ml of 8 M urea-0.1 M sodium pyrophosphate, pH 9.0, and allowed to react with maleic anhydride. The reaction was performed as described by Butler et al.(13,14) by adding about 60 mg of maleic anhydride. The pH was kept at about 9.0 by additions of 1 M NaOH.

The maleylated sample was trasferred to a Sephadex G-100 column (2.5 x 110 cm), equilibrated with 0.05 M ammonium bicarbonate. The elution was carried out with the same solvent at 15 ml/hr (with collection of 5 ml fractions). The absorbance of the fractions at 280 nm was monitored with a LKB 8300 Uvicord II, and the radioactivity was followed on aliquots from every second tube in a liquid scintillation spectrometer.

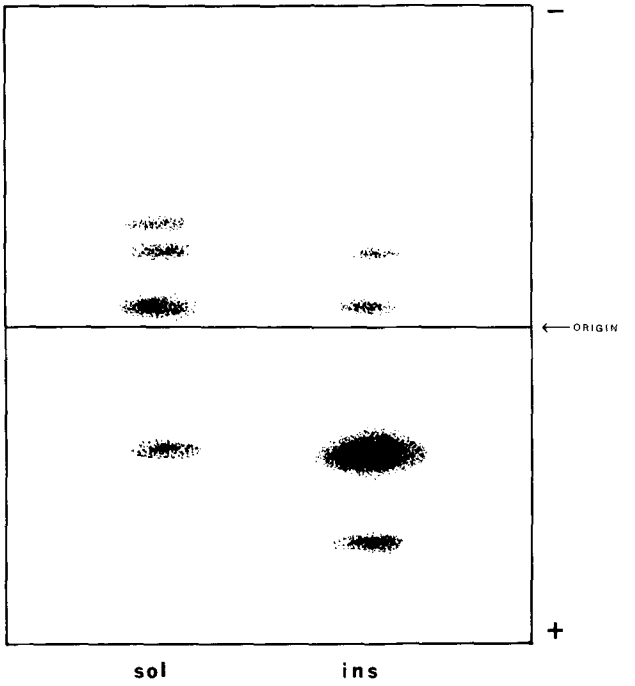

Fig.1 - Radioautogram of radioactive peptides produced by tryptic digestion of the soluble (sol) and the insoluble (ins) fractions obtained after CNBr cleavage of yeast alcohol dehydrogenase inactivated by iodo (2-^{14}C)acetate and fully carboxymethylated. Electrophoresis was performed in pyridine-acetic acid buffer at pH 6.5.

The elution pattern is shown in fig.2.

The fractions, containing the radioactive peak, were pooled and freeze-dried. The dry residue was dissolved in 0.05 M ammonium bicarbonate and digest with trypsin for 4 hours at 37°C. The tryptic digest was applied to a column of Sephadex G-50 (2.5 x 110 cm), equilibrated with 0.05 M ammonium bicarbonate. The elution was carried out with the same buffer at 20 ml/hr (with collection of 5 ml fractions). Optical density at 280 nm and radioactivity were followed as described above. One single peak of radioactivity, containing the 80% of the radioactivity of the insoluble fraction, emerged from the column (Fig.2).

The more radioactive fractions were pooled and demaleylated as described by Butler et al.(13,14). A single radioactive peptide was obtained by high voltage paper electrophoresis at pH 6.5.

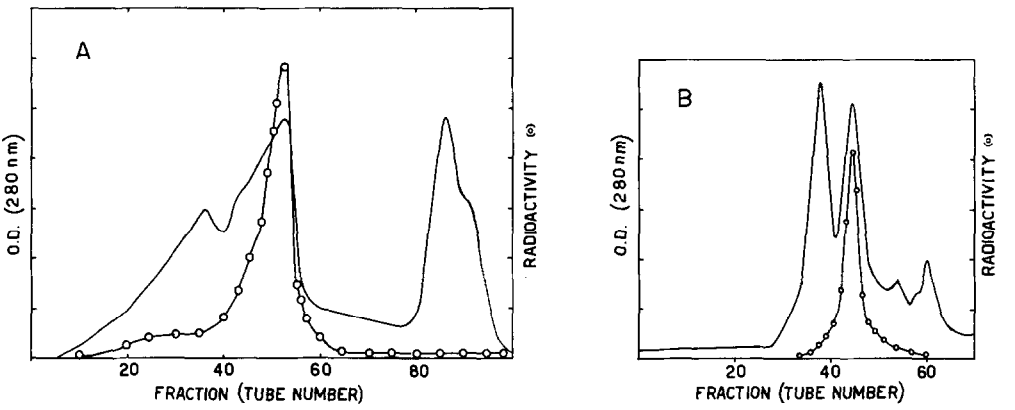

Fig.2 - Isolation of the main radioactive peptide from iodoacetate inactivated yeast alcohol dehydrogenase. After inactivation the sample was fully carboxymethylated and subjected to CNBr cleavage. The digest was divided in two fractions as described in the text; the insoluble fraction was maleylated and applied into a Sephadex G-100 column (2.5 x 110 cm) equilibrated with 5 x 10^{-2} M ammonium bicarbonate. The elution was carried out with the same solvent at 15 ml/hr with collection of 5 ml fractions (A). The main radioactive fractions were pooled and subjected to tryptic hydrolysis. The tryptic digest was then fractionated by Sephadex G-50 gel filtration. The column (2.5 x 110 cm) was eluted with 5 x 10^{-2} M ammonium bicarbonate at 20 ml/hr with collection of 5 ml fractions (B).

Amino acid sequence of radioactive peptide - The amino acid composition of the radioactive peptide was determined on a Beckman Model 120 C Amino Acid Analyzer after hydrolysis of the sample in evacuated glass tube (6 N HCl, 24 hours, 105°C) (15). The estimated amino acid content was : CMCys(2), Asp(1), Ser(1), Pro(1), Gly(1), Ala(2), Val(1), Phe(1), Lys(1). No residue of tryptophan was detected by using Ehrlich test (16) in the unhydrolyzed peptide. The N-terminous, determined by dansyl procedure (17), was proline.

Both the whole peptide and the two peptides obtained by its tryptic digestion were subjected to sequence analysis by the dansyl-Edman procedure (18). Dansyl-amino acid were identified by two-dimensional thin layer chromatography on polyamide sheets using the solvents reported by Woods and Wang (19). The sequences were :

Pro-Ala-Gly-Ala-Lys-^{14}CMCys-CMCys-Ser-Asp-Val-Phe

T_1 T_2

T

The C-terminal phenylalanine is probably formed by a chymotryptic-like cleavage. This peptide could represent the N-terminal part of a larger peptide produced by CNBr cleavage.

DISCUSSION

Using approximately the same experimental conditions employed by Harris (1), we isolated a radioactive peptide which revealed an amino acid sequence different from that found by Harris after inactivation of yeast alcohol dehydrogenase with iodo(2-^{14}C)acetate. Even if the loss of activity was about 90% with an incorporation of 2.2-2.4 moles of iodo (2-^{14}C)acetate per mole of yeast alcohol dehydrogenase, we found more that one radioactive peptide. The peptide, that we followed and sequenced, was clearly the more radioactive, having the 80% of radioactivity of the insoluble fraction which contained about the 70% of the initial radioactivity.

The peptide, that we sequenced, was not found by Jornvall in the amino-terminal region of the molecule (20); however this worker has recently identified in the primary structure of yeast alcohol dehydrogenase a peptide with the same sequence (H.Jornvall, unpublished result).

It is not easy to fully explain the different localization of ^{14}C-labeling. The inactivation of yeast alcohol dehydrogenase with iodoacetate was carried out in similar experimental conditions, except for the pH, that was 7.0 in our experiments and 7.5 in Harris'work. Recently Belke et al.(21) have demonstrated that the inactivation of yeast alcohol dehydrogenase with thiol reagents (iodoacetate, iodoacetamide, butyl isocyanate) involves different cysteine residues in an amount strongly determined by the pH at which the reaction is carried out; in particular the radioactive iodoacetate is incorporated preferably in the Harris'peptide at pH 7.5, but at lower pH values the labeling increases in the butyl isocya-

nate-reacting peptide and in other peptides, that were considered as different segments of the Harris'peptide produced by heterogeneous peptic cleavages (21).

The present results and the works of Harris (1), Dickinson (8), Twu et al.(9) and Belke et al.(21) indicate than the yeast alcohol dehydrogenase contains more than one reactive thiol group and the enzyme molecule exposes to various thiol reagents different cysteine residues with the varying of the experimental conditions and of the employed reagents. Modifications of thiol groups in different points of the protein give rise to conformational changes, which impair more or less the enzymatic activity.

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