Metal-catalyzed Oxidation of Fe$^{2+}$ Dehydrogenases

CONSENSUS TARGET SEQUENCE BETWEEN PROPANEOL OXIDOREDUCTASE OF ESCHERICHIA COLI AND ALCOHOL DEHYDROGENASE II OF ZYMOMONAS MOBILIS*

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We have studied two enzymes of a newly described family of dehydrogenases with high sequence homology, 1,2-propanediol oxidoreductase of Escherichia coli and alcohol dehydrogenase II of Zymomonas mobilis. These enzymes perform their metabolic role under anaerobic conditions; in the presence of oxygen, they show a very similar inactivation pattern by a metal-catalyzed oxidation system. Titration of histidine residues with diethyl pyrocarbonate showed one histidine residue less in the oxidized enzymes. Comparison of subtilisin peptide maps of active and inactivated enzymes showed a difference in one histidine-containing peptide, the sequence of which is YNTPH$^{77}$GVAN for propanediol oxidoreductase and YNLPH$^{77}$GV for alcohol dehydrogenase II. This histidine residue lies 10 residues away from a proposed metal-binding site, HEP$^{20}$XXXHH$^{21}$, necessary to explain a site-specific free radical mechanism. The three histidine residues here described are strictly conserved in all enzymes of this family. In this report we propose that histidine 277 is a target for oxidation by a metal-catalyzed oxidation system and that this modification leads to the irreversible inactivation of both enzymes.

In Escherichia coli, the anaerobic metabolism of fucose requires the activity of propanediol oxidoreductase to reduce the l-lactaldehyde to l-1,2-propanediol, a fermentation product, using NADH as a cofactor (1). The enzyme, which is induced by the methylpentose regardless of the respiratory conditions of the culture, remains fully active in the absence of oxygen. In the presence of oxygen, propanediol oxidoreductase becomes irreversibly inactivated by metal-catalyzed oxidation (MCO)1 (2, 3).

Zymomonas mobilis is obligately ethanologenic and requires a fermentable sugar for energy and growth even in a rich medium (4, 5). The pyruvate-to-ethanol pathway is indispensable in Z. mobilis for NADH oxidation. The ethanol pathway consists of a single pyruvate decarboxylase and two isoenzymes of alcohol dehydrogenase (I and II) (5). Alcohol dehydrogenase II is the dominant enzyme during fermentation, converting acetaldheyde to ethanol using NADH as a coenzyme (6, 7).

Propanediol oxidoreductase and alcohol dehydrogenase II are iron-dependent enzymes of a recently described family of dehydrogenases (8, 9) similar in sequence but not homologous to either short chain or long chain zinc-containing alcohol dehydrogenase enzymes (10). Here we present evidence for the inactivation of alcohol dehydrogenase II mediated by MCO, which is similar to that found for propanediol oxidoreductase (3).

Further comparative studies of these two enzymes in terms of specific amino acid modification and peptide mapping lead us to propose a target consensus sequence for a metal-catalyzed oxidation process.

EXPERIMENTAL PROCEDURES

Materials

The strains used were an E. coli K12, strain ECL1 (11), also known as E15 (12) (kindly provided by E. C. C. Lin) and Z. mobilis (ATCC 29191).

Enzyme Assays

Propanediol oxidoreductase activity was measured as described in Boronat and Aguilar (13), except that glycolaldehyde was used instead of lactaldehyde as a substrate (14), monitoring the oxidation of NADH to NAD by the loss of absorbance at 340 nm. Alcohol dehydrogenase II activity was measured by the rate of NADH formation at 340 nm, using ethanol as substrate, as described in Mackenzie et al. (15).

Protein Determination

Protein concentration was determined by the Bradford (16) method, using bovine serum albumin as standard.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Electrophoresis was performed according to Laemmli (23), using 12% acrylamide as resolving gel. Proteins were stained with Coomassie Blue R-250 (24).

Purification of Enzymes

Purification of Propanediol Oxidoreductase—Anaerobically or aerobically growing cells were harvested at logarithmic phase, resuspended in four times their wet weight with 0.05 M Tris-HCl buffer, pH 7.5, containing 2.5 mM NAD, and disrupted by sonication. Following centrifugation at 100,000 × g (13), ammonium sulfate was added to the supernatant to 40% saturation. After equilibration, the precipitate was collected by centrifugation at 15,000 × g for 30 min and dissolved in 20 ml of 0.05 M Tris-HCl buffer, pH 7.5. This fraction was applied to an

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‡ The abbreviations used are: MCO, metal-catalyzed oxidation; DEPC, diethyl pyrocarbonate; HIC, hydrophobic interaction chromatography; HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; MES, 4-morpholinoethanesulfonic acid.
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Ultragel AcA44 gel filtration column (5 x 70 cm) equilibrated with the last buffer and eluted with a flow rate of 3 ml/min. Fractions with propanediol oxidoreductase activity were pooled and applied to a DEAE-15HR (Waters) preequilibrated with the same buffer. Proteins were eluted in 50 min with a linear gradient (0–0.25 M NaCl in Tris-HCl buffer). Protein ion-exchange chromatography was performed with a flow rate of 5 ml/min. Samples with activity were pooled and dialyzed against Tris buffer.

Preparations of propanediol oxidoreductase appeared as a single band in SDS-PAGE. The specific activity was 35 unit/mg for the anaerobic enzyme and 10 unit/mg for the aerobic enzyme.

Purification of Alcohol Dehydrogenase II—Cells were grown to the stationary phase in a rich medium as described by Mackenzie et al. (15), harvested, and resuspended in 0.02 M MES-KOH buffer, pH 6.5, containing 5 mM ammonium ferrous sulfate (four times their wet weight) and disrupted by sonication. Following centrifugation at 100,000 x g, the supernatant was applied to a Sephacryl S-200 gel filtration column (4 x 75 cm), equilibrated with the same buffer, and eluted with a flow rate of 2 ml/min. Fractions with alcohol dehydrogenase II activity were pooled and applied to a DEAE-15HR ion-exchange column. Proteins were eluted with a linear gradient (0–0.15 M NaCl in MES buffer), at a flow rate of 5 ml/min. Samples with activity were pooled and dialyzed against iron-containing MES buffer. Preparations of alcohol dehydrogenase II appeared as a single band in SDS-PAGE. The specific activity was 750 unit/mg.

**In Vitro Enzyme Inactivation**

Inactivation of propanediol oxidoreductase and alcohol dehydrogenase II was performed by the following procedure. For aerobic studies, 2 ml of crude extracts containing 15 mg of protein/ml were incubated in a rotary shaker at 120 rpm for 1 h. For anaerobic studies, samples were introduced into conical glass vials, fitted with a Teflon-lined rubber septum, and preflushed with nitrogen for 5 min before the incubation vial was tightly capped. For *in vitro* inactivation of purified enzymes the reaction mixture consisted of 1.5 mM of enzyme (1 mg/ml), 25 mM FeCl₃, and 35 mM ascrobate (3).

**Chemical Modification**

*Modification by 5,5'-Dithio-bis-(2-nitrobenzoic Acid)—*Titration of sulfhydryl groups of enzymes with 5,5'-dithiobis-(2-nitrobenzoic acid) was performed at 25 °C in a 50 mM Tris/HCl buffer, pH 8.2, in a final volume of 1 ml. The reaction was triggered by the addition of 0.1 ml of a 10 mM solution of 5,5'-dithio-bis-(2-nitrobenzoic acid) in methanol to 0.9 ml of a solution of propanediol oxidoreductase enzyme (50–100 µg). The rate of reaction was monitored by the change in absorbance at 412 nm with time. A molar extinction coefficient (ε) of 14,150 M⁻¹ cm⁻¹ (17, 18) was used to calculate the number of sulfhydryl groups titrated.

*Modification by 2,4,6-Trinitrobenzenesulfonic Acid—*The chemical modification of enzymes with 2,4,6-trinitrobenzenesulfonic acid was carried out at 40 °C for 3 h in a 0.5 mM borate buffer, pH 9.5. The reaction was initiated by the addition of a diluted solution of 2,4,6-trinitrobenzenesulfonic acid as described by Tuengler and Pfeiderer (19). The number of modified lysine residues was calculated from the molar absorption differences at 342 nm using a molar extinction coefficient (ε) of 14,400 M⁻¹ cm⁻¹ (20).

*Modification by Diethyl Pyrocarbonate (DEPC)—*The chemical modification of purified enzymes with DEPC was carried out at 25 °C in a 50 mM phosphate buffer, pH 6.0. The stoichiometry of the formation of N-carboxyethylysine residues was determined by the increase in absorbance at 243 nm using an extinction coefficient (ε) of 3,200 M⁻¹ cm⁻¹ (20).

The reaction was started by the addition of an ethanolic solution of DEPC (final concentration of 0.5 mM) to a solution of 0.01 mM enzyme (propanediol oxidoreductase or alcohol dehydrogenase II) in 1 ml, as described by Topham and Daizel (21), and concluded when the absorbance maximum at 243 nm was obtained. The residual enzyme activities were determined at different times after the addition of DEPC.

*Reaction with Hydroxylamine—*The DEPC-inactivated enzyme was incubated at 25 °C with either 0.15 or 0.4 mM hydroxylamine at pH 6.8. The time course of removal of the carboxethylysine group from N-carboxyethylhistidine residues was followed by continuously recording the change in absorbance at 243 nm.

**Carbonyl Group Determination—**The carbonyl content of native and denatured propanediol oxidoreductase was determined by the dinitrophenylhydrazone method (typically from 250 to 600 µg of protein) according to Levine et al. (22). Results are reported as means of triplicate assays for each sample.

**Susceptibility to Proteolysis**

Susceptibility to proteolysis by subtilisin was determined by incubating 50 µg of purified propanediol oxidoreductase with 0.25 µg of subtilisin at 37 °C as described previously (3).

**Hydroporphic Interaction Chromatography (HIC)**

Hydroporphic interaction chromatography was performed on a phenyl column (Shodex PH-814, 8 x 75 mm). Samples of native in *vivo* or in *vitro* inactivated propanediol oxidoreductase were dissolved in buffer A consisting of 0.1 M sodium phosphate, 1.5 M ammonium sulfate, pH 7.0. Buffer B was 0.1 M sodium phosphate, pH 7.0. The flow rate was 1.0 ml/min, and a linear gradient was applied from 70 to 100% of buffer B for 30 min and maintained for 20 min. The chromatogram was monitored at 280 nm.

**HPLC Peptide Mapping and Sequencing**

For subtilisin digestion, the samples (200–500 µg of each active and inactivated enzyme) were denatured by adding three volumes of 8 M guanidinium chloride in 0.05 M phosphate buffer, pH 7.0, and subsequently incubated for 15 min at 30 °C. The solution was then diluted with the same buffer to a final guanidinium chloride concentration of 2 M, subtilisin was added (5% of the mass of substrate), and the mixture was incubated at 30 °C for 2 h, with continuous stirring.

The peptides obtained were separated by reverse phase chromatography using a HPLC Waters system and a Delta-Pak C18 column (3.9 x 15 mm, pore size, 300 A). Peptides were eluted on a gradient of 0.1% trifluoroacetic acid in acetonitrile/water (10:90) as solvent A, and 0.1% trifluoroacetic acid in acetonitrile/water (60:40) as solvent B. For each run, the flow rate was 0.8 ml/min, and the gradient slope was 0–60% of buffer B for 60 min. The chromatogram was monitored using a uv/vis recorder (λ = 214 nm). Manually collected fractions were dried in a Speedvac concentrator (Savant). Peptides were sequenced using a 470A Applied Biosystems gas phase sequencer.

**Results**

**Propanediol Oxidoreductase**

**Determination of Histidine Residues—**Studies of MCO-inactivated enzymes demonstrate that modification is highly specific and affects one or fewer amino acid residues. Some authors suggest that lysine, cysteine, or histidine may be the most common oxidized residues and thus responsible for enzyme inactivation (25, 26).

The His content estimated in our experiments with DEPC revealed the presence of 3 ± 0.4 titratable residues in the active propanediol oxidoreductase and 2 ± 0.3 residues in the propanediol oxidoreductase inactivated in *vivo* or in *vitro*. Thiol groups measured by 5,5'-dithiobis-(2-nitrobenzoic acid) showed no change in any form of the enzyme. Determination of Lys residues with 2,4,6-trinitrobenzenesulfonic acid also revealed no differences among them (not shown).

**Inactivation of Propanediol Oxidoreductase by DEPC—**DEPC inactivated propanediol oxidoreductase in a linear time-dependent manner at pH 6.0. No loss in enzyme activity was detectable in control incubations performed using ethanol instead of DEPC. The rate of inactivation was initially first order with respect to any given concentration of DEPC (Fig. 1). A plot of the pseudo-first order rate constants of inhibition (kₒba) versus DEPC concentration was linear (Fig. 1, inset), giving a second order rate constant of 116 M⁻¹ min⁻¹. These results indicated that the modification reaction between the enzyme and the reagent was a simple bimolecular reaction.

A double logarithmic plot of the half-times of inactivation against reagent concentration according to the studies of Levy et al. (27) gave a reaction order (stoichiometry) of 0.9. This demonstrates that covalent modification of a single histidine residue inactivated propanediol oxidoreductase.

The participation of other residues was excluded on the basis of the following data. (i) The UV difference spectrum of DEPC-modified propanediol oxidoreductase showed an increase in ab-
sorbance only at 243 nm, characteristic of N-carbethoxyhistidine residues (28). The complete absence of spectral decrease at 278 nm eliminates the possible formation of O-carbethoxytyrosine. (ii) The incubation of DEPC-treated enzyme with 0.4 M hydroxylamine, pH 6, resulted in the total recovery of the initial 243 nm absorbance. The reversibility of this reaction eliminates the N-carbethoxylation of lysine residues (20). However, no enzyme activity was recovered at this stage. Similar results have been reported for several other enzymes (29-31).

To localize the essential histidine residue, substrate protection against DEPC modification was assayed. Table II shows that the substrates, 1-lactaldehyde or propanediol, and NADH prevented one histidine residue from being modified. This protection is consistent with a higher remaining activity (55-60%) after treatment with DEPC, in clear contrast with a control experiment without protection (25%).

**Carbonyl Group Content**—A commonly reported structural modification caused by MCO is the introduction of carbonyl groups onto amino acid side chains. These groups can react with 2,4-dinitrophenylhydrazine to form protein hydrazone derivatives (22). By using this property, the protein carbonyl content was determined on the purified active enzyme and in vivo and in vitro inactivated propanediol oxidoreductase. The results showed an increased content of these groups in the two inactivated forms of propanediol oxidoreductase (0.64 ± 0.015 and 0.85 ± 0.015 mol of dinitrophenylhydrazine/mol of protein for in vivo and in vitro inactivated propanediol oxidoreductase, respectively) when compared with the control enzyme (0.11 ± 0.012 mol of dinitrophenylhydrazine/mol of protein).

**Separation of Native and Oxidized Forms of Propanediol Oxidoreductase** by HIC—To obtain clear results for the following experiments, the separation of native and oxidized forms of propanediol oxidoreductase was crucial, because previous experiments showed no clear differences between them in peptide mapping.

Fig. 2A shows the HIC separation of propanediol oxidoreductase purified from aerobic or anaerobic cultures. Anaerobic propanediol oxidoreductase eluted as a single peak (I), whereas aerobic propanediol oxidoreductase eluted as a double peak. The main peak (II) represents the inactive form, and the secondary peak, which coelutes with the anaerobic form, is presumably the remaining active form responsible for the low levels of activity seen in the aerobic extracts (2). The inset shows that the apparent molecular weights of both peaks are identical and that fragmentation, which has been shown in several proteins during the inactivation process (32), does not occur.

Fig. 2B shows the time course of exposure of peak I protein to the ascorbate-iron oxidation system resolved by HIC. The in vitro inactivation of the enzyme progressively changed the conformation of the enzyme to a more hydrophobic form (peak II), which eluted with the same retention time as the inactivated enzyme in vivo.

This separation allowed us to test the loss of activity in parallel to the susceptibility to digestion by subtilisin. The inactivation process does not match the shift from the native form to the more hydrophobic one (Table III). The conformational change was slower than the inactivation. In addition, peak II protein showed a higher susceptibility to subtilisin degradation.

**Peptide Mapping of Native and Inactivated Forms of Propanediol Oxidoreductase**—To identify the target of the MCO system, we studied the subtilisin peptide maps obtained from native and inactivated (in vivo and in vitro) forms. Repetitive differences were observed only when the samples used were those obtained after HIC (peak I, native, and peak II, inactivated form). The peptide mapping of native propanediol oxidoreductase is shown in Fig. 3A. The corresponding map of the form inactivated in vitro is shown in Fig. 3B (the same results were obtained with the enzyme inactivated in vivo). While one peak eluted at 19.50 min in the native preparation, no such peak was detected in the oxidized forms of the enzyme. The peptide was purified and subjected to Edman degradation. The sequence obtained was YNTPH277GVAN.
in control experiments. As shown in Table II, addition of 1 mM NADH to the cuvette of reaction clearly prevented the inactivation, and only 0.5 His/mol of subunit enzyme was modified after treatment with DEPC.

**Carbonyl Group Content**—The method used for propanediol oxidoreductase was applied to quantify the carbonyl content of the purified active and inactivated alcohol dehydrogenase II. A marked increase in hydrazones derivatives was detected in the inactivated form (0.95 ± 0.015 mol of dinitrophenylhydrazine/mol of protein) when compared with the native one (0.11 ± 0.015 mol of dinitrophenylhydrazine/mol of protein).

**HPLC Peptide Mapping and Sequencing**—The peptide maps of alcohol dehydrogenase II enzyme were obtained using subtilisin with native and inactivated forms. A difference was observed in a peptide eluting at 31.9 min in the map corresponding to the native form (Fig. 4A) but not in the modified form (Fig. 4B). The purification of this peptide and the determination of amino acid sequence gave YNLPH27GV.

**DISCUSSION**

Previous results indicated that propanediol oxidoreductase is synthesized in an active form irrespective of the respiratory conditions of the culture. The enzyme is rapidly inactivated in the presence of oxygen. Pure propanediol oxidoreductase preparations were inactivated in vitro by aerobic incubation in the presence of Fe3+ and ascorbate (MCO system). The oxidized form is degraded faster than the native form (3).

Treatment with DEPC shows that one histidine residue is involved in the loss of activity of propanediol oxidoreductase. The use of 5,5'-dithiobis-(2-nitrobenzoic acid) and 2,4,6-trinitrobenzenesulfonic acid eliminates the possibility that thiol groups or lysine, respectively, might be involved in the inactivation process. Furthermore, this essential histidine must be located at the active center of the enzyme, because protection with substrate or cofactor clearly prevented inactivation. Three histidines were modified using the inhibitor/enzyme ratio of 50 (Table II). One of these residues, protected from modification by substrates (lactaldehyde or propanediol) or coenzyme (NADH), is important for the function of the enzyme, because most of the activity was retained under these conditions.

HIC, which separates the active from inactive forms of propanediol oxidoreductase, provides a reasonable explanation of the low levels of enzyme activity displayed in aerobic cultures (2), because 20% of the pure aerobic enzyme preparation eluted with the same retention time as the native anaerobic enzyme (Fig. 2A). Moreover, the in vitro modification of pure propanediol oxidoreductase reveals a gradual change in the overall hydrophobicity, which is the same as that observed in the aerobic enzyme. Previous results (3) have shown that aerobic enzyme was more susceptible to proteolytic degradation; this is in good agreement with the results presented in Table III, because the more hydrophobic (inactivated form) is proteolyzed 2.5 times faster than native enzyme.

By comparing the results obtained with alcohol dehydrogenase II and those obtained with propanediol oxidoreductase (described here and previously published Refs. 2 and 3), several
similarities are found. (i) Both enzymes are inactivated in the presence of oxygen in the medium, in good agreement with previous observations of Neale et al. (33) with respect to the high sensibility of alcohol dehydrogenase II enzyme to loss of activity during the purification. We have shown that the presence of coenzyme in crude extracts prevented the inactivation. (ii) Alcohol dehydrogenase II, like propanediol oxidoreductase, is inactivated by an MCO system, which means that a site-specific-inactivating process is taking place. This inactivation was prevented by catalase or the coenzyme but not by superoxide dismutase. (iii) The use of DEPC reagent allows titration of histidine residues in the different forms of alcohol dehydrogenase II enzyme. As shown in Table I, the inactivated enzyme has one titratable histidine residue less than the control enzyme, which implies that one histidine residue is oxidized, and this modification avoids the formation of the N-carbethoxyhistidine derivative. In the presence of NADH, which protects the catalytic site, only 0.5 histidine residue/mol of subunit is modified. In this case, the inactivation is largely prevented. This is another similarity with the propanediol oxidoreductase, in which one histidine residue is responsible for the inactivation process.

In terms of obtaining clear differences in peptide mapping, the use of propanediol oxidoreductase protein eluted from HIC (peaks I and II) was crucial. We identified one peptide present only in the native form, but we failed to localize its modified form in the peptide mapping obtained from the inactivated enzyme. The sequence of the His-containing peptide is YNTPH277GVAN. In the case of the alcohol dehydrogenase II, the HIC separation of the modified form was not required to obtain clear differences in the modified peptide maps. Again, like the propanediol oxidoreductase, a parent peptide is lost upon oxidative inactivation of alcohol dehydrogenase II. The sequence obtained from this purified peptide was YNLPH277GV, which is almost equal to that obtained from propanediol oxidoreductase. Consequently, we conclude that His277 is a consensus target for oxidative modification.

The failure to isolate the modified peptides may be explained by the fact that a histidine residue can be converted to an asparaginyl residue by metal-catalyzed oxidation (32), which might serve as a new target site for subtilisin attack, and so, the protease would split the peptide in two fragments and make it difficult to locate it in the peptide map.

Propanediol oxidoreductase and alcohol dehydrogenase II are included in a newly described family of dehydrogenases (8, 9), which show a high degree of sequence identity (propanediol oxidoreductase shows 41.7% identity with alcohol dehydrogenase II). These proteins are not homologous to either short chain or long chain zinc-containing alcohol dehydrogenase enzymes (10). Walter et al. (9) have indicated that six proline residues are strictly conserved in this new family of alcohol dehydrogenases, suggesting possible similarities in the three-dimensional structures of these proteins; they have also emphasized the presence of four strictly conserved His residues, three of which are in a 15-amino acid stretch (Table IV). One of these residues is present in the sequenced peptides, and the other two are located in an α-helix (predicted by applying the Chou & Fasman program for analysis of secondary structure) and possibly involved as metal binding ligands (H²60-X-X-H²67). As described by Higaki et al. (35) such a motif possesses two correctly positioned imidazole rings, which together might chelate a metal ion.

By bearing in mind that a histidine residue and a metal ion are both involved in the catalytic mechanism of dehydrogenases, the above mentioned 15-amino acid stretch could be a
portion of the active center. In this center the three conserved histidine residues would be in a pocket where metal, substrate, and coenzyme are in close contact (36, 37). Consistently, the enzymes using Fe$^{2+}$ or another metal ion with two oxidation states and having this target sequence are likely to be inactivated in the presence of oxygen and by generation of free radicals, which attack amino acid residue(s) located in the active center.

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