Investigation of the metal-binding site of the metalloenzyme NAD\(^+\) : 2-oxidoreductase (glycerol dehydrogenase) EC 1.1.1.6 from Bacillus stearothermophilus

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Glycerol dehydrogenase has now been isolated from a number of sources including mammals, bacteria and fungi. Possible functions in vivo have been reported, e.g. xerotolerance in fungi and algae (Ben-Amotz & Avron, 1973), biosynthesis and/or degradation of substituent side chains of vitamin B12 in Escherichia coli (Kelly & Deckker, 1985) and the sole means of energy and carbon source (May & Sloan, 1981).

With the exception of the membrane-bound GDH of Gluconobacter indurans which requires pyrroloquinoline quinone as a co-factor (Ameyama et al., 1985) all known glycerol dehydrogenases are soluble and pyridine nucleotide dependent. These have been classified into three types dependent upon the site of oxidation on glycerol (C1 or C2) and whether NAD\(^+\) or NADP acts as the co-factor (Kong et al., 1985): type I: glycerol: NAD\(^+\) 2-oxidoreductase (EC 1.1.1.6); type II: glycerol: NAD\(^+\) oxidoreductase (EC 1.1.1.72 specific for glycerol and trioses; EC 1.1.1.21 broader based aldehyde or aldose reductase); and type III: glycerol: NADP\(^+\) 2-oxidoreductase (EC 1.1.1.56).

GDH from Bacillus stearothermophilus has been shown to be of type I, being NAD\(^+\) specific, exhibiting no reductive activity towards glycaldehyde and no oxidative activity to 1,3-propanediol or polyol sugars.

SDS-PAGE experiments have shown that the subunit is around \(M_s\) 42,000 and together with gel-filtration experiments these data support the hypothesis of a tetrameric structure.

GDH has been found by radiolabelling using iodine-131-acetic acid, performic acid oxidation and amino acid analysis, and chemical cleavage techniques to have only one cysteine residue per subunit. The protein was subjected to chemical cleavage on the N-terminal side of the cysteine by cyanololation using 2-nitro-5-thio-cyanobenzoate (Jacobson et al., 1973). This results in the formation of two peptides, one a blocked peptide beginning with a modified cysteine residue and another peptide which has the N-terminal sequence of the native protein. This latter peptide was detected by the method described by Doolittle & Jue (1985) and had an \(M_s\) of 24,000 measured by SDS-PAGE. Experiments using the same method, but employing the reagent cyanogen bromide to cleave the protein on the carboxy side of methionine residues, showed that there was also only one copy of this amino acid per polypeptide. This result was confirmed by amino acid analysis.

Preparation for several hours to chelating agents such as EDTA or 8-hydroxyquinoline leads to loss of activity of some GDH enzymes. More immediate inactivation of GDH from Bacillus megaterium has been demonstrated with 0.5 mm-1-phenoanthraniline (Scharshmidt et al., 1983) which is thought to directly chelate to the metal which is still bound to the enzyme (Vallee et al., 1958). In the case of the GDH from B. stearothermophilus, the nature of the metal ion has yet to be unambiguously identified. However, removal of the metal to give apo-GDH results in complete loss of activity which can be regained by the addition of certain divalent cations, e.g. Zn\(^{2+}\), Cd\(^{2+}\) in the assay medium.

The presence of a bound metal in GDH might be expected to stabilize the protein against denaturation. Native holo-GDH was found to be stable at 60\(^\circ\)C. After removal of the metal, the half-time for denaturation at 60\(^\circ\)C was 3 min, however, the apo-enzyme was still stable at 50\(^\circ\)C (for at least 20 min). Therefore the metal alone does not confer heat stability. Its protein structure is also important.

The binding site for the metal was investigated using side-chain specific chemical modifications. Modification of any residue involved in metal binding or in close proximity might be expected to prevent reactivation of apo-GDH in metal-containing assay buffer.

Dithyl pyrocarbonate (DEP) is reportedly specific for histidine residues at pH 6 (Dominici et al., 1985) and its modification can be followed at 240 nm (\(6.3\times10^4\)). This modification is freely reversible using 1 m-hydroxylamine, whereas regeneration of free lysine from carboethoxylated lysine is not.

A total of four to five histidine residues in the holo-enzyme was found to be modified with 1 mm-DEP resulting in a 10% loss of activity. With apo-GDH, six to seven histidine residues could be modified with total loss of activity. Addition of metal to the apo-enzyme before modification (20 \(\mu\)m-Zn\(^{2+}\)) protected the enzyme from inactivation and reduced the number of histidine residues modified to four to five. The statistical method of Tsou (1962) was used to analyse the number of essential histidine residues. This indicated that two residues were essential and that the modification of either of them leads to complete inactivation. This analysis is based upon the assumption that all residues are of equal reactivity with the reagent. Addition of hydroxylamine to 1 m, was found to reverse the change in absorption at 240 nm and resulted in restoration of the potential activity of modified apo-GDH. The metal protects two histidine residues which when modified result in the loss of activity. It is possible that the two histidines may be acting as ligands to the metal. The presence of cofactor 40 \(\mu\)m-NADH (\(K_d\) 2 \(\mu\)m) did not protect against histidine modification.

Methyl methane thiosulphonate (MMTS) is specific for cysteine residues and its modification is freely reversible on addition of \(\beta\)-mercaptoethanol (Bloxham et al., 1979). Addition of 10 mm-MMTS to holo-GDH resulted in no loss of activity whereas apo-GDH lost all activity. Again the presence of the cofactor NADH (20 \(\mu\)m) was unable to protect against the modification and the modified apo-GDH was still able to bind NADH. It is possible therefore that the cysteine residue may be acting as a third ligand to the metal.

Abbreviations used: GDH, glycerol dehydrogenase; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; DEP, diethylpyrocarbonate; MMTS, methyl methane thiosulphate.


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The partial purification of cytochrome P-450 from the digestive gland of the common mussel

Mytilus edulis

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The enzyme is localized primarily in the endoplasmic reticulum of the digestive gland (hepatopancreas) and can be collected in the microsomal fraction (microsomes). We report here a procedure for the partial purification (8-fold) of this enzyme from digestive gland microsomes by solubilization with sodium cholate, ammonium sulphate precipitation followed by affinity chromatography with 8-amino-octyl Sepharose 4B (Pharmacia) and ion-exchange chromatography with DEAE-Sephercel (DEAE-S) (Table 1).

Microsomes prepared by the method of Livingstone & Farrar (1984) were resuspended in 100 mM-phosphate buffer pH 7.6 using 20% (w/v) glycerol, 1 mM-EDTA, 1 mM-DTT and 0.2% (v/v) Emulgen 911. The column was washed with this buffer and eluted with a KCl gradient (0-500 mM). One major cytochrome P-450 peak eluted at 10-40 mM-KCl and

Table 1. The partial purification of cytochrome P-450 from the digestive gland of M. edulis

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Specific content (pmol·mg protein)</th>
<th>Total content (nmol)</th>
<th>Purification factor</th>
<th>Yield (%)</th>
<th>Ratio P-450 (\Delta A 418-490) peak</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude microsomes</td>
<td>51.2</td>
<td>38.8</td>
<td>1.0</td>
<td>100</td>
<td>0.4</td>
</tr>
<tr>
<td>Microsomes solubilized in 1.3% cholate</td>
<td>31.6</td>
<td>18.1</td>
<td>0.6</td>
<td>45.5</td>
<td>0.1</td>
</tr>
<tr>
<td>35-65% (NH₄)₂SO₄ precipitate</td>
<td>41.3</td>
<td>7.5</td>
<td>0.8</td>
<td>18.9</td>
<td>0.2</td>
</tr>
<tr>
<td>8-amino-octyl S4B affinity step</td>
<td>108</td>
<td>3.0</td>
<td>2.1</td>
<td>7.5</td>
<td>1.1</td>
</tr>
<tr>
<td>DEAE-Sephercel ion-exchange step</td>
<td>417.0</td>
<td>2.4</td>
<td>8.2</td>
<td>5.9</td>
<td>∞</td>
</tr>
</tbody>
</table>

Abbreviation used: DTT, dithiothreitol.

Cytochrome P-450 and the mixed-function oxidase system in M. edulis (Livingstone, 1985).

Ammonium sulphate fractionation was carried out on the supernatant by collecting the precipitate at 35-65% saturation at pH 7.0 according to the method of King et al. (1984). The precipitate was resuspended in a reduced volume of 0.015 M-phosphate buffer at pH 7.0 containing 20% (w/v) glycerol, 1 mM-EDTA, 1 mM-DTT and 0.3% (v/v) sodium cholate, and dialysed against 30 volumes of this buffer for 12 h. Insoluble material was removed by centrifugation (100,000 g, 50 min) and the supernatant applied directly to an affinity column of 8-amino-octyl Sepharose 4B, pre-equilibrated with 0.01 M-phosphate buffer pH 7.0 containing 20% (w/v) glycerol, 1 mM-EDTA, 1 mM-DTT and 0.3% (v/v) cholate. The equilibration buffer minus DTT (100 ml) was run through in a washing step, and cytochrome P-450 was then eluted as a relatively sharp peak with the wash buffer containing 0.2% (v/v) Emulgen 911 (a non-ionic detergent). The cytochrome P-450 recovery for this step is high (40-50%) and there is a purification for the step of more than 2-fold (range 2.5-3.5-fold). The affinity stage provides an effective means of separating the P-450 from the NADPH-dependent cytochrome c (P-450) reductase activity.

Eluted fractions containing cytochrome P-450 were then combined and the total volume reduced by ultrafiltration at 4°C (membrane, 30 kDa cut-off). The pH of the resulting cytochrome P-450-rich fraction was then adjusted to pH 7.6 by dialysing for 4 h against 0.01 M-phosphate buffer pH 7.7 containing 20% (w/v) glycerol and 0.2% (v/v) Emulgen 911. The dialysate was applied to a DEAE-Sephercel ion-exchange column, pre-equilibrated with 0.01 M-phosphate pH 7.6 containing 20% (w/v) glycerol and 0.2% (v/v) Emulgen 911. The column was washed (3 volumes) with this buffer and eluted with a KCl gradient (0-500 mM). One major cytochrome P-450 peak eluted at 10-40 mM-KCl and