D- and L-6-Hydroxynicotine Oxidase, Enantiozymes of *Arthrobacter oxidans*

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D- and L-hydroxynicotine oxidase were obtained in homogeneous form and compared with regard to molecular weight, subunit structure, reaction mechanism, and specificity. The most striking differences between these enantiozymes are the type of coenzyme binding and the reactivity towards artificial two-electron acceptors.

The flavoproteins, D- and L-6-hydroxynicotinamide oxidase, are induced when *Arthrobacter oxidans* is grown in the presence of D- and L-nicotine, respectively. Both enzymes are absolutely stereospecific, the enantiomeric substrate acting as a competitive inhibitor. They form the same product, [(6-hydroxy-2-pyrrolidinyl) - (γ-N-methylaminopropyl)] ketone ("ketone"), from either D- or L-6-hydroxynicotine by an apparently identical mechanism. The term enantiozymes is proposed for pairs of enzymes having these properties.

The D- and L-6-hydroxynicotine oxidases were obtained in homogeneous form. As summarized in the Table they differ in molecular weight, subunit structure, reactivity towards two-electron acceptors, FAD-content, and coenzyme binding, the D-specific enzyme having one mole of covalently bound FAD per mole of protein. FAD as the prosthetic group of D-6-hydroxynicotinamide oxidase was identified by spectroscopy and AMP-determination, that of the L-specific enzyme by paper chromatography of the dissociated coenzyme and by reactivation of the apoenzyme by FAD³.

Oxygen appears to be the physiological electron acceptor for both enzymes. One-electron acceptors such as cytochrome c and ferricyanide as well as methylene blue and 2,6-dichlorophenol-indophenol do not reoxidize the reduced L-6-hydroxynicotine oxidase.

![Diagram](image-url)

Table. D- and L-6-Hydroxynicotine oxidase from Arthrobacter oxidans.

| 6-hydroxynicotinamide oxidase | mol. W. | f/0 | poly-peptide chains/mole | FAD bound (absorption maxima) | turnover number (substrate) | reaction towards electron acceptors | FAD/ | Km (substrate) |
|-------------------------------|---------|-----|--------------------------|-------------------------------|-----------------------------|------------------------------------| FAD | (M) |
| D-                            | 53 000  | 1.26| 1                        | covalently (272, 355, 450, S475) | 1190 (pH 9.2; 30°C) | 5·10⁻³ M - (stimul.) + |
| L- (ref. 4)                   | 93 000  | 1.22| 2                        | non-covalently (273, 370, 443, S463) | 1760 (pH 7.2; 29°C) | 2·10⁻³ M - (inhib.) + |

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oxidase. Moreover, methylene blue was shown to be a strong inhibitor of the overall reaction. One-electron acceptors are also ineffective with the D-specific enzyme; however, methylene blue and 2,6-dichlorophenol-indophenol are able to reoxidize the reduced D-6-hydroxynicotine oxidase. In the presence of these dyes the rates of the overall process are higher than those with oxygen.

Anaerobic titrations of both enantiozymes with their respective substrates produced a steady transition from the oxidized to the reduced form. Spectral evidence of a semiquinoid intermediate stage could not be obtained. This was seen, however, after illumination of the enzymes in the presence of EDTA.

It remains to be elucidated whether and to what extent reactivity towards artificial two-electron acceptors of D- and L-6-hydroxynicotine oxidase and the type of coenzyme binding are a consequence of the respective quaternary structure of these proteins.

References


Covalently Bound Flavin in D-6-Hydroxynicotine Oxidase from Arthrobacter oxidans

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Flavoprotein, Covalently bound FAD, 8α-(N3-histidyl)-riboflavin

D-6-hydroxynicotine oxidase contains 1 mole of FAD covalently bound to one mole of enzyme. To identify the covalent linkage between FAD and protein, an amino acid derivative of riboflavin (HNO-flavin) was isolated and purified. It was obtained from flavin peptides by hydrolysis with 6 N HCl at 95 °C or with aminopeptidase M. The riboflavin derivative had the spectral characteristics of 8α-substituted flavins. It showed a pH-dependence of fluorescence with a pK of 4.65 and 86% quenching at pH 7. In thin layer chromatography it was identical with 8α-(A-3-histidyl)-riboflavin. Hydrolysis of HNO-flavin in 6 N HCl at 125 °C liberated 1 mole of histidine per mole of flavin as shown by amino acid analysis. Since FAD is the coenzyme of D-6-hydroxynicotine oxidase, these results are taken as evidence that this enzyme contains 8α-(N3-histidyl)-flavin-adenine-dinucleotide in the active center.

Unusual Abbreviations: HNO-flavin, riboflavin attached to one amino acid of D-6-hydroxynicotine oxidase; His-riboflavin, 8α-histidyl-riboflavin.

D-6-Hydroxynicotine oxidase from Arthrobacter oxidans, catalyzing the oxidative cleavage of D-6-hydroxynicotine to (6-hydroxypyridyl-(3))- (y-N-methyaminopropyl)-ketone, contains one mole of FAD per mole of enzyme. Methods known to reversibly dissociate the flavin coenzyme from the apoprotein failed to release the coenzyme. Treatment of the enzyme with 5% trichloroacetic acid resulted in a supernatant without any absorbance in the visible range whereas the precipitate displayed the typical flavin spectrum. Tryptic digestion of the purified enzyme yielded a fluorescent product, migrating differently than FAD in paper chromatography and high voltage electrophoresis. These results were taken as evidence for the presence of a covalently bound flavin in D-6-hydroxynicotine oxidase.

To identify the covalent linkage between FAD and the apoprotein, an amino acid derivative of riboflavin (HNO-flavin) was isolated from flavin peptides either by hydrolysis in 6 N HCl at 95 °C in vacuo for 12 h or with aminopeptidase M containing prolinase. The aminopeptidase digest was then incubated in 10% trichloroacetic acid in order to hydrolyze the flavin dinucleotide to the mononucleotide and subsequently at pH 5.7 with acid phosphatase for dephosphorylation. The resulting HNO-flavin was purified by preparative thin layer.