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-hydroxynicotinic oxidase and the type of coenzyme binding are a consequence of the respective quaternary structure of these proteins.

1 K. DECKER and H. BLEEG, Biochim. biophysica Acta [Amsterdam] 105, 313 [1965].

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-hydroxynicotinic 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-(N3-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-hydroxynicotinic oxidase, these results are taken as evidence that this enzyme contains 8α-(N3-histidyl)-flavin-adenine-dinucleotide in the active center.

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chromatography on cellulose with n-butanol-acetic acid-H$_2$O 2:1:2 and 8:1:91, respectively. It showed the following characteristics:

Compared to the spectrum of riboflavin, HNO-flavin showed at pH 7 a hypsochromic shift of the peak at 370 nm to 346 nm. It was further displaced to 341 nm at pH 2.2; this shift reflects an additional protonation of the amino acid. In 6 N HCl the absorption band of riboflavin at 395 nm was displaced to 370 nm in HNO-flavin. This spectral behaviour is characteristic for 8α-substitution of the flavin nucleus.

The fluorescence intensity of HNO-flavin was pH-dependent. The maximal intensity at pH 3.2 was quenched by 86% at pH 7.0. The pK of fluorescence quenching was 4.65. This suggests a secondary or tertiary amino group attached to the flavin. For synthetic 8α-histidyl-riboflavin, containing predominantly the N-3-isomer, a pK of 4.7 was found.

Hydrolysis of HNO-flavin in 6N HCl at 125 °C for 20 h resulted in the release of histidine, glutamic and aspartic acid. Glutamate and aspartate as components of HNO-flavin are excluded by the fluorescence data. The release of 1 mole of histidine per mole of HNO-flavin in 80% yield is in agreement with all the data presented for HNO-flavin.


<table>
<thead>
<tr>
<th>Properties</th>
<th>HNO-flavin</th>
<th>His-riboflavin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absorption spectrum in 6 N HCl (λ$_{max}$)</td>
<td>370 nm,</td>
<td>370 nm,</td>
</tr>
<tr>
<td>Fluorescence-quenching at pH 7 as compared to</td>
<td>265 nm</td>
<td>265 nm</td>
</tr>
<tr>
<td>pH 3.2</td>
<td>86%</td>
<td>94%</td>
</tr>
<tr>
<td>pK of fluorescence-quenching</td>
<td>4.65</td>
<td>4.7</td>
</tr>
<tr>
<td>nmoles histidine per nmole liberated by acid</td>
<td>0.8</td>
<td>0.6</td>
</tr>
<tr>
<td>hydrolysis</td>
<td>0.27</td>
<td>0.28</td>
</tr>
<tr>
<td>Rp of N-3-isomer</td>
<td>—</td>
<td>0.33</td>
</tr>
</tbody>
</table>

HNO-flavin, obtained with aminopeptidase, migrated identically with the N-3-isomer of synthetic 8α-histidyl-riboflavin in thin layer chromatography on cellulose with n-butanol-acetic acid-H$_2$O (2:1:2).

A comparison of synthetic 8α-histidyl-flavin and HNO-flavin is given in the Table. Since FAD is the coenzyme of D-6-hydroxynicotinic oxidase, these results are taken as evidence that this enzyme contains 8α-(N-3-histidyl)-flavin-adenin-dinucleotide in the active center.

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4 S. Ghisla and P. Hemmerich, FEBS Lett. 16, 229 [1971].
5 S. Ghisla, U. Hartmann, and P. Hemmerich, Angew. Chemie 82, 669 [1970].