4 of the flavin nucleus. These findings support the general idea that (4a)C=N(5) is the main redox reaction center of the flavin nucleus. Furthermore, it is obvious that the minimum requirement for irreversible additions of the above type is the "substrate" grouping HC=CH2 and that such inhibitions might be a general principle in flavoprotein chemistry.


Nitroalkanes as Reductive Substrates for Flavoprotein Oxidases

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Stoichiometry, proton abstraction, kinetic mechanism

Nitroalkanes have been found to be general reductive substrates for D-amino acid oxidase, glucose oxidase and L-amino acid oxidase. These enzymes show different specificities for the structure of the nitroalkane substrate.

Each enzyme differs somewhat in its specificity for nitroalkanes. At pH 8.1, with substrates in their equilibrium states of ionization, the imolecular constant for flavin reduction is in the order nitromethane < nitroethane < 1-nitropropane < 2-nitropropane for D-amino acid oxidase. This order of reactivity is also approximately true for L-amino acid oxidase, whereas with glucose oxidase the order is almost completely reversed. The nitroalkanes reduce free FAD photochemically, but we have observed no reactivity in the dark.

The overall stoichiometry of nitroalkane oxidation is analogous to that for the natural substrates. The D-amino acid oxidase reaction is accurately described by Eqn. 1.

\[ \text{RCH}_2\text{NO}_2 + \text{OH}^- + \text{O}_2 \rightarrow \text{RCHO} + \text{NO}_2^- + \text{H}_2\text{O}_2. \]  

(1)

In the case of glucose oxidase at pH 5.0, Eqn. 1 does not entirely account for the stoichiometry since only about 0.5 moles each of \( \text{H}_2\text{O}_2 \) and \( \text{NO}_2^- \) are formed. The remaining nitrogen is largely accounted for as \( \text{NO}_3^- \), with a small fraction appearing as 1-dinitroalkane.
The kinetics of nitroalkane oxidation depend very much on whether the neutral or anionic form of the substrate is used. We have recently described the kinetics of oxidation of neutral nitromethane by D-amino acid oxidase. Very similar kinetic patterns are observed in the case of neutral nitroethane and glucose oxidase. In both reactions the bimolecular interaction of substrate with oxidized enzyme (E0) is completely rate-determining in turnover. The pH dependence of this process in the case of glucose oxidase and nitroethane is very similar to that observed with natural substrates and gives evidence for the participation of a basic group with an apparent pK value of about 5.5. However, the nitroethane anion is an incomparably better reductive substrate than the neutral substrate and flavin reduction is sufficiently rapid to allow the detection of a first order kinetic process following the interaction of O2 with reduced enzyme. As a consequence, the steady state turnover data give ping pong patterns. Surprisingly, the pH dependence of the bimolecular interaction of -C2H4NO2 with E0 is precisely opposite to that observed for C2H5NO2 and the natural sugar substrates; that is, the rate of flavin reduction increases 10-fold with each pH unit decrease. It is also remarkable that, after correction for the fraction of inactive aci-substrate present at low pH values, the rate of flavin reduction by nitroethane anions is significantly greater than that observed with D-glucose, the most reactive natural substrate. These findings are consistent with the hypothesis that general base-catalyzed proton abstraction from the neutral nitroalkane is an obligatory process in catalysis and that the nitroalkane anion, which lacks this proton, does not interact effectively with the enzyme at pH values where the enzyme base (possibly carboxylate) is negatively charged. Stopped-flow measurements of both the half-reactions and steady state kinetic data, support the following kinetic mechanism (Eqn. 2), at pH 5.0 for the oxidation of nitroethane by glucose oxidase. All of the rate constants have been evaluated.

\[ \text{EX} + \text{O}_2 \rightarrow \text{E}^\cdot \rightarrow \text{ES} \rightarrow \text{E}^- + \text{NAD}^+ \]

The formation of the semiquinone (E\(\cdot\)) from ES is kinetically insignificant in the case of oxidation of the nitroethane anion.

Our efforts to test the hypothesis that the substrates of flavoprotein oxidases interact covalently with the flavin nucleus have centered on the observation that D-amino acid oxidase is slowly inactivated during the oxidation of nitromethane. We have been able to isolate, after inactivation, a modified form of FAD which has an absorption spectrum very similar to that of inactivated holo-enzyme and from which, in aqueous solution in the presence of O2, oxidized flavin can be regenerated by heating to 80° at pH 8.3. We hope that characterization of the modified coenzyme will contribute to the understanding of chemical events in flavoprotein catalysis.
