Inactivation of a Flavoprotein, Lactate Oxidase, by an Acetylenic Substrate*

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SUMMARY

The acetylenic substrate, 2-hydroxy-3-butynoic acid, inactivates lactate oxidase from Mycobacterium smegmati. When inactivated lactate oxidase is separated into flavin and apoprotein components, the apoprotein is reactivatable by exogenous FMN while the flavin has been converted to a fluorescent, reduced species stable to autoxidation. Incubation between substrate and flavin is lost in the inactivation process which involves adduct for the apoprotein, indicating that the -hydrogen must be lost in the inactivation process which involves adduct formation between substrate and flavin.

Certain acetylenic amines inhibit mitochondrial monoamine oxidase, a flavoprotein (1-3). When monoamine oxidase is inhibited by N-methyl-N-(propynyl)-benzylamine (pargyline), an undissociable, possibly covalent complex is formed between enzyme and inhibitor (3). It is not known whether the inactivator reacts with the flavin or the apoenzyme. These results, as well as the inactivation of -OH-decanoy-thioester-dehydrase (4), a non-flavoprotein, by an acetylenic substrate prompted us to investigate further the reaction of acetylenic substrates with flavoproteins. If they form adducts with the flavin, a study of these reactions could be generally useful in elucidating the occurrence and structure of flavin-substrate adducts, which may occur as intermediates in oxidations catalyzed by flavoproteins (5-7). We have found that a primary amine, 2-propynylaniline, inactivates monoamine oxidase and appears to do so by interaction with the flavin. Similarly, 2-OH-3-butynoic acid inactivates lactate oxidase, a flavoprotein, which catalyzes the following reaction:

$$H$$

$$(L)-R-COO^- + 0_2 \xrightarrow{E-FMN} RCOO^- + CO_2 + H_2O$$

This report deals primarily with the latter reaction since it has been studied more extensively than monoamine oxidase.

MATERIALS AND METHODS

2L-2-Hydroxy-3-butynoic acid was prepared by a published procedure (8, 9). 3L-2-Hydroxy-3-[2-3H]butynoic acid was prepared by equilibrating the acid with lactate dehydrogenase and [4-3H]NADH in the presence of a small amount of NAD. 2L-2-Hydroxy-3-[4-3H]butynoic acid was prepared by equilibration with HDO at pH 11 for 30 min. 3-Butynoic acid was a gift from Mr. Louis Riceberg of the Chemistry Department, Brandeis University. Lactate oxidase was purified from Mycobacterium smegmatis (10). Enzyme activity was measured by oxygen uptake with an oxygen electrode apparatus. Protein was measured by the Lowry method (11) or by the extinction coefficients reported by Sullivan (11).

RESULTS AND DISCUSSION

2L-2-Hydroxy-3-butynoate is a substrate ($K_m = 2 \, \text{mm}$) for lactate oxidase as well as an inactivator. Fig. 1 shows that in the presence of 2L-2-OH-3-butynoic acid, oxygen consumption ceases before the oxygen supply is exhausted. The initial rate of oxygen consumption is 1.05 pmoles per min per mg of enzyme, but ceases when 19.5 nmoles of oxygen are consumed (Curve 1). At this point the enzyme is inactive. Addition of L-lactate...

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FIG. 1. Oxygen uptake with 2L-2-hydroxy-3-butynoate. Incubations 1, 2, and 3 contained 100 mm imidazole-CI, pH 7.0, and 20 mm 2L-2-hydroxy-3-butynoate. Incubation 4 contained the imidazole buffer and 50 mm L-lactate. Each solution was equilibrated with air before being placed in the oxygen electrode cell whose volume was 0.3 ml. Enzyme was added at the point indicated by the first arrow; 27 mg were added to each incubation containing acetylenic substrate while 3 mg were added to that containing lactate. With L-lactate as substrate, the specific activity of the enzyme was 22.6.
an excitation very similar to the absorption spectrum. The final llavin spectrum, indicating that the Hnvin was not autoxi-
flavin is nor1fluorescent, the inactivated enzyme exhibits a strong
species (Fig. 2). The aerobic inactivation yielded the same
probably reflects a process that occurs at the active site.
droxy-3-butynoate, calculated on the assumption that only the
inactivation; it is a competitive inhibitor (not shown). Thus,
incubation with 0.8
02-saturated solution.
addition (Curve S) resulted in the uptake of an additional 19.5
of oxygen. Prior to complete inactivation 35 turnovers
occur in 2070 02.saturated solution and 165 turnovers in 100%
O2-saturated solution.
Inubcation of lactic oxidase with 2-0H-3-butynoic acid leads to a
time-dependent inactivation. When 50 µg of enzyme were
incubated with 0.8 mM DL-2-OH-3-butynoate, a half-time of in-
activation of 115 min was observed. On the other hand, the
closely related compound 3-butynoic acid produces no irreversible
inactivation; it is a competitive inhibitor (not shown). Thus,
the inactivation process is intimately connected to the ability of
2-hydroxy-3-butynoic acid to function as a substrate and probably reflects a process that occurs at the active site.
Under anaerobic conditions a stoichiometric amount of 2-hy-
droxy-3-butynoate, calculated on the assumption that only the
form is reactive, completely inactivates the enzyme. The in-
activated coenzyme-enzyme complex contained a reduced-type
flavin species (Fig 9). The aerobic inactivation yielded the same
final flavin spectrum, indicating that the flavin was not autoxi-
dizable after inactivation. Whereas the active oxidized enzyme-
flavin is nonfluorescent, the inactivated enzyme exhibits a strong
yellow fluorescence with emission maximum at 505 nm and with
an excitation very similar to the absorption spectrum.
This result suggested that inactivation occurred by interaction
with the flavin coenzyme rather than the apoprotein. To test
this possibility, 5 mg of enzyme (0.1 µmole, specific activity =
14.4) were inactivated with 20 µmoles of DL-2-hydroxy-3-butynoate
for 15 min at room temperature to produce greater than
99.5% inactivation. The enzyme was separated from substrate
by Sephadex gel filtration and resolved into flavin and apoprotein
by acid ammonium sulfate treatment (12, 13). The apo-
protein recovered from the inactivated as well as untreated holo-
enzymes can be reactivated to the same extent with exogenous
FMN, proving that the acetylenic acid causes inactivation by
reacting with the coenzyme.
To test for covalent attachment of the acetylenic compound
to the flavin, the enzyme was inactivated and subjected to gel
filtration on Sephadex G-25. The enzyme-bound flavin was then
released either by heating for 2 min at 80° or by adding 1
volumes of methanol at room temperature. The spectrum of the
released flavin (Fig. 2) shows no absorbance at 450 nm but
differs from the enzyme-bound flavin. When the untreated
holoenzyme was treated similarly, oxidized FMN was released.
These data suggest that combination of the acetylenic substrate
and flavin coenzyme occurred to produce a nonautoxidizable
form of the reduced coenzyme.
Experiments with tritium-labeled acetylenic acid were carried
out to identify some of the structural changes which occur during
inactivation. When lactate oxidase was treated with 2-hy-
droxy-3-[2-3H]butynoate and isolated by gel filtration, the in-
activated protein contained no 3H, indicating that the substrate
must lose its α-hydrogen before the inactivation occurs. When
2-hydroxy-3-[4-3H]butynoate was used, the inactivated enzyme
after Sephadex contained 1 mole of 3H per mole of active site.
After resolving the modified flavin from the apoprotein, all of
the tritium was associated with the flavin. The acetylenic
linkage originally present in the substrate is no longer present in
the isolated flavin inhibitor adduct: the 2-hydroxy-4-[3H]butyno-
ate exchanges 100% of its 3H on standing in water at pH 11
for 45 min, while the 4-[3H]-modified flavin inactivator adduct lost
only 8% of its 3H in this treatment.
These results, together with the spectral data, prove that an
adduct is formed between enzyme-bound FMN and 2-0H-
3-butynoic acid. Formation of this adduct leads to enzyme
inactivation. Based on the data reported here and previously
(14), the minimal reaction sequence proposed is shown below.
Formation of the intermediate E* from holoenzyme and 2-OH-
3-butynoic acid involves the loss of the α proton from the acety-
lenic acid. This is consistent with experiments with α- and
L-amino acid oxidase (14), as well as lactate oxidase,4 which
indicate that abstraction of the substrate α-proton as a proton
occurs early in the reaction sequence between flavoenzyme and
substrate. E* can then react with oxygen to form the reaction
products or it can undergo a nonoxidative process to form the
substrate-2-OH-butynoic acid adduct, which is catalytically inactive
(E**). The partitioning of E* between two reaction pathways
is consistent with the observation that the rate of inactivation
varies inversely with oxygen concentration.
The formation of the complex between FMN and 2-0H-3-

1The failure of 132 mM L-lactate to elicit oxygen uptake at this point
is not attributable to simple competitive inhibition by the acetylenic
substrate. In a separate incubation, when enzyme was added to a
mixture of 90 mM L-2-OH-butynoate and 132 mM L-lactate, there was
a rapid uptake of oxygen and the inactivation process was much slower.
1The product from the O2-dependent reaction of 2-OH-3-butynoate
has not yet been identified. However, there is no detectable H2O2
formed, suggesting that O2 is reduced all the way to H2O as is the case
in reaction of lactate. By analogy then, if H2O is formed, one would
expect oxidative decarboxylation to generate 2-propionic acid (pro-
ionic acid) and carbon dioxide.

butynoic acid involves loss of substrate α-hydrogen and the acetylenic linkage, which could occur as follows.

1. Rearrangement of the acetylenic compound to the allene after removal of the α-hydrogen. The allene could then react further to produce the inactive flavin adduct. Such a reaction sequence would be analogous to that reported for the inactivation of β-OH-decanoyl-thioester dehydrase by acetylenic thioesters (4).

2. Addition of a nucleophilic center of the flavin, such as N-5 or C-4 of the reduced flavin, to the acetylenic linkage.

Recently we have observed that another flavoprotein, pig liver mitochondrial monoamine oxidase, is inactivated and labeled by a primary amine, [3-3H]propynylamine. It may well be that propynylamine forms an adduct with the flavin. Acetylenic substrate analogues may therefore be generally useful as inactivators of flavoproteins. Elucidation of adducts formed between these inactivators and flavins will provide important insights into the general mechanism of action of flavins in enzymatic reactions. Recently the inactivation of d-amino acid oxidase by nitromethane has been reported (15); the inactivation probably involves adduct formation between inactivator and flavin.

A number of adducts between flavins and acetylenic compounds have been obtained through photolytic reactions in which it is hypothesized that addition of N-5 to the acetylenic linkage has occurred. Insufficient information is available at this time to determine whether the photolysis products and the enzymic products are similar. The structure of the adduct between FMN and the acetylenic acid in lactate oxidase is now under intensive investigation.

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Inactivation of a Flavoprotein, Lactate Oxidase, by an Acetylenic Substrate
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