Preparation of Aldehyde Oxidase in Its Native and Deflavo Forms

COMPARISON OF SPECTROSCOPIC AND CATALYTIC PROPERTIES*

(Received for publication, January 21, 1974)

UMBERTO BRANZOLI† and VINCENT MASSEY

From the Department of Biological Chemistry, The University of Michigan, Ann Arbor, Michigan 48104

SUMMARY

Aldehyde oxidase (EC 1.2.3.1) has been purified by a modification of a previously reported procedure and the FAD prosthetic group has been removed by treatment with calcium chloride and calcium acetate. The deflavo enzyme so obtained is devoid of \( N^1 \)-methylnicotinamide oxygen reductase activity but can be reconstituted by a short incubation with FAD.

The different activities of native and deflavo enzymes using oxygen, cytochrome c, potassium ferricyanide, nitro blue tetrazolium, nitro blue tetrazolium, dichlorophenolindophenol, and FAD as electron acceptors and \( N^1 \)-methylnicotinamide as substrate are compared.

The similarities between xanthine oxidase and aldehyde oxidase have been recognized since the early studies on these enzymes (see Refs. 1 and 2 for recent reviews of the literature). Our interest in aldehyde oxidase arose largely from recent findings with xanthine oxidase that a persulfide group was concerned in enzyme catalysis (3) and that the availability of a catalytically active deflavo enzyme was very helpful in elucidating patterns of electron flow in this multifactor-containing enzyme. This paper describes the preparation and properties of aldehyde oxidase in its native and deflavo forms; the accompanying paper documents the evidence for an active site persulfide residue in this enzyme (4).

MATERIALS AND METHODS

\( N^1 \)-Methylnicotinamide oxidase activity was routinely tested according to Felsted (5). The conversion of NMN to its 6-pyridone was measured by following the increase in optical density at 300 nm after addition of the enzyme to a 1-ml final volume reaction containing: 0.05 M potassium phosphate, pH 7.8, 0.3 mM EDTA, 5 mM NMN, and 10 \( \mu \)g of catalase, air-equilibrated at 25°C. To convert the observed absorbance changes into international units of activity, i.e. micromoles of substrate oxidized per min, the extinction coefficient for the 6-pyridone of 4.23 \( \times 10^3 \) cm\(^{-1} \) M\(^{-1} \) was used. Protein concentration was determined by the biuret method (6) and the molybdenum content by the diithiol method of Ringerly (7). Iron was determined as Nitrilotriphosphate chelate after trichloroacetic acid denaturation (8). FAD was determined by measurement of its absorbance at 450 nm after liberation by trichloroacetic acid and neutralization with phosphate. The extinction coefficient for FAD was taken as 11.3 \( \times 10^3 \) cm\(^{-1} \) M\(^{-1} \) (9).

Calcium phosphate gel was prepared by the method of Swingle and Tiselius (10). Glass-distilled water was used for all reagents and EDTA was present in all buffers at a concentration of 0.3 mM. A Gilford spectrophotometer was used for the determination of all enzyme activities and spectra were routinely measured with a Cary model 17 or 118 recording spectrophotometer. NMN, NBT, DCIP, and Cyt. c were obtained from Sigma; catalase (bovine liver) and O-phenanthroline from Calbiochem; and 3,4-dimercapto-1-octanol from Aldrich. The source material for enzyme purification was frozen mature rabbit livers obtained from Pel-Freeze, Biologicals, Inc., Rogers, Arkansas.

Purification Procedure

The enzyme was purified mainly according to the procedure of Rajagopalan et al. (11) with some minor modifications.

**Step 1: Homogenate and Heat Treatment**—The rabbit livers were homogenized for 2 min in 5 volumes of 0.05 M phosphate, pH 8.0, containing 1 mM EDTA and rapidly heated to 62°C in a water bath set at 85°C. After 2 min the homogenate was chilled to 15°C in a glycol bath set at -20°C and centrifuged at 13,000 \( \times \) g for 40 min.

**Step 2: Ammonium Sulfate Fractionation**—To the supernatant from Step 1 solid ammonium sulfate was added to 0.50 saturation (313 g per liter) and after 30 min the precipitated protein was collected by centrifugation at 13,000 \( \times \) g for 40 min.

**Step 3: Acetone Fractionation**—The precipitate from the previous step was resuspended in 0.05 M phosphate, pH 7.8, containing 0.3 mM EDTA and the solution was clarified by centrifugation. It was then chilled to 0°C and diluted to a concentration of about 42 mg per ml. Then acetone, previously chilled at -20°C, was added under vigorous stirring to 40% saturation. A large amount of denatured protein was separated from the enzyme by centrifugation at 4,000 \( \times \) g for 5 min and the supernatant was chilled to -15 to -20°C. This is done to reduce the loss of enzyme at this very critical stage. Then acetone, previously chilled at -25°C, was added to 50% concentration. The precipitate enzyme was collected by centrifugation and resuspended in cold distilled water containing 0.3 mM EDTA. The pH was adjusted at 7.8 with 2 N KOH and ammonium sulfate was added to 0.50 saturation. The precipitate was collected by centrifugation at 20,000 \( \times \) g for 15 min and resuspended in cold distilled water containing 0.3 mM EDTA and the pH again adjusted at 7.8 with 2 N KOH.

**Step 4: Calcium Phosphate Gel Adsorption and Elution**—Enough calcium phosphate gel to bind almost all of the enzyme was added
from the gel with two washes of 250 ml each of 0.05 M phosphate, water containing 0.3 mM EDTA and the enzyme was finally eluted.

The gel was collected by centrifugation at 400 X g for 5 min. The gel was washed three times with 400 ml each time of cold glass-distilled water containing 0.3 mM EDTA and the enzyme was finally eluted was washed three times with 400 ml each time of cold glass-distilled water containing 0.3 mM EDTA. The traces of ammonium sulfate were separated from calcium phosphate gel were pooled and solid ammonium sulfate was added to a final concentration of 60%. After 30 min the precipitated protein was collected by centrifugation and resuspended in a small volume of 5 mM Tris-glycine buffer, pH 7.8, containing 0.22 M KCl. The fractions showing the characteristic visible spectrum of the enzyme were pooled and concentrated with an Amicon ultrafiltrator equipped with a XM-50 ultrafilter. At this point in the purification the enzyme is stable to filtration and has a ratio of 280:450 ranging from 7 to 9.

Step 6: DEAE-Sephadex Chromatography—The two eluates from calcium phosphate gel were pooled and solid ammonium sulfate added to a final concentration of 60%. After 30 min the precipitated protein was collected by centrifugation and resuspended in a small volume of 5 mM Tris-glycine buffer, pH 7.8, containing 0.3 mM EDTA. The enzyme was immediately applied to a DEAE-Sephadex A-50 column (1.9 X 50 cm) equilibrated with 5 mM Tris-glycine buffer, pH 7.8, containing 0.3 mM EDTA and 0.15 M KCl. Elution was started with a linear gradient of 1 liter of buffer containing 0.15 M KCl and 1 liter containing 0.22 M KCl. The fractions showing the characteristic visible spectrum of the enzyme were pooled and concentrated with an Amicon ultrafiltrator equipped with a XM-50 ultrafilter. At this point in the purification the enzyme is stable to storage and dialysis and has a ratio of 280:450 ranging from 7 to 9 and a specific activity of 1.8 i.u. per mg.

Step 6: Sephadex G-200 Chromatography—This last step has been introduced to separate the enzyme from contaminating proteins with low molecular weight. For a standard preparation a column (1 X 60 cm) equilibrated with 0.05 M phosphate, pH 7.8, was found to be 0.4 mM.

Preparation of Deflavo Aldehyde Oxidase

Rajagopalan et al. (14) reported the resolution of the flavin from aldehyde oxidase by dialysis against 3 M KI. During such treatment the enzyme indeed lost its oxygen reductase activity but also showed a concomitant decrease for DCIP and methylene blue reductase activities suggesting either denaturation or loss of other cofactors (or both). In addition attempts to recombine the deflavo enzyme with FAD were without success.

Previously Komai et al. (15) reported the preparation of a functional deflavo milk xanthine oxidase by treatment with 2 M CaCl2. Such enzyme was still able to reduce ferricyanide when incubated with xanthine and also recovered its oxygen and Cyt. c reductase activities after preincubation with FAD. A slight modification of this second procedure led us to the reversible removal of the dodecyl sulfate gel electrophoresis, the minimum molecular weight per mole of enzyme-bound FAD is calculated to be 173,000. This value is somewhat higher than that originally estimated by other authors (11, 12) from sedimentation measurement, but is close to the value reported for milk xanthine oxidase (13).

Table I

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume</th>
<th>Protein concentration (mg/ml)</th>
<th>Total protein (mg)</th>
<th>Total activity (iu)</th>
<th>Specific activity (iu/mg)</th>
<th>Yield %</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Homogenate and heat treatment</td>
<td>3,520</td>
<td>-</td>
<td>-</td>
<td>562</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>B. Ammonium sulfate precipitation</td>
<td>137</td>
<td>54.2</td>
<td>7,350</td>
<td>544</td>
<td>0.076</td>
<td>97</td>
</tr>
<tr>
<td>C. Acetone fractionation, 40-50%</td>
<td>94</td>
<td>38.2</td>
<td>3,591</td>
<td>540</td>
<td>0.153</td>
<td>96</td>
</tr>
<tr>
<td>D. Calcium phosphate gel eluate</td>
<td>30</td>
<td>34.2</td>
<td>1,026</td>
<td>432</td>
<td>0.420</td>
<td>77</td>
</tr>
<tr>
<td>E. DEAE Sephadex chromatography</td>
<td>4</td>
<td>20</td>
<td>79.7</td>
<td>143</td>
<td>1.8</td>
<td>25.5</td>
</tr>
<tr>
<td>F. Sephadex G200 chromatography</td>
<td>4</td>
<td>16.3</td>
<td>65</td>
<td>140</td>
<td>2.2</td>
<td>25</td>
</tr>
</tbody>
</table>

Content of Molybdenum and Nonheme Iron

The enzyme as purified above contains in addition to FAD, as previously reported (1, 5), molybdenum and nonheme iron. In our preparations an average of 0.87 g atom of molybdenum and 4.1 g atom of nonheme iron per mole of enzyme-bound FAD were found.

Specific Activity

Under the conditions specified by Felsted and Chaykin (5), the AFR value at 25° of the purified enzyme has been found routinely in the range 40 to 46.2 This corresponds to a specific activity of 2.0 to 2.2 i.u. per mg of protein and a turnover number of 320 to 380 moles of substrate oxidized per min per mole of enzyme-bound FAD. The apparent Km for NMN in air-equilibrated 0.05 M phosphate, pH 7.8, was found to be 0.4 mm.

The AFR value is defined as the change in ABW per min in the AFR value at 25° of the purified enzyme has been found routinely in the range 40 to 46.
FIG. 1 (left). Comparison of the absorption spectra of native and deflavo aldehyde oxidases (5.7 mg per ml) in 0.05 M phosphate, pH 7.8. The lower curve shows the calculated difference spectrum. The right vertical axis shows the extinction coefficient (m⁻¹ cm⁻¹) based on the FAD content of the native enzyme.

FIG. 2 (right). Absorption spectra of deflavo aldehyde oxidase (7.9 mg per ml) in the oxidized and reduced state. The lower curve is the calculated difference spectrum. Conditions, anaerobic in 0.05 M phosphate, pH 7.8. The reduced state was obtained by the anaerobic addition of 5 mM NMN.

Fig. 3. Oxygen reductase activity. Left, plot of the reciprocal turnover number versus reciprocal oxygen concentration at four different NMN concentrations. Right, plot of the reciprocal turnover number versus reciprocal NMN concentration. Circles, native enzyme without added FAD; triangles, deflavo enzyme in presence of 1 x 10⁻⁴ M FAD. The reactions were carried out in 0.05 M phosphate, pH 7.8, at 25°. The experiments with deflavo enzyme plus FAD were in air-equilibrated buffer.

RESULTS

Absorption Spectrum

Fig. 1 shows the spectra of native and deflavo aldehyde oxidases. The spectrum of the deflavo enzyme is quite similar to that of xanthine oxidase (15) and other iron-sulfur proteins (16) with absorption maxima at 460, 425, and 320 nm. At wavelengths greater than 550 nm the spectrum is identical with that of the native enzyme. Fig. 1 also shows the calculated difference spectrum between native and deflavo enzyme; this is typical of a flavin from aldehyde oxidase. Two main observations were of great help in improving the yield. First, calcium acetate reduces the extent of denaturation and second, FMN (produced by hydrolysis of FAD) even at low concentrations, inactivates the enzyme unless light is excluded.

Solutions of aldehyde oxidase (4 to 6 mg per ml) in 0.1 M Tris, pH 8, containing 0.3 mM EDTA were incubated in the dark at 25° for 3 to 4 hours in the presence of 1.7 M calcium chloride and 0.7 M calcium acetate. The solution was then passed through a Bio-Gel P-2 column (1 x 30 cm) equilibrated with 0.1 M Tris, pH 8, containing 9 mM cysteine. The fractions containing the deflavo enzyme were pooled, concentrated, and dialyzed for 24 hours versus 0.05 M phosphate, pH 7.8. The yield of the deflavo enzyme usually varied from 60 to 70% of the starting material.
# Table II

Comparison of catalytic activities of native and deflavine enzymes

<table>
<thead>
<tr>
<th>Acceptor</th>
<th>Concentration range (mM)</th>
<th>Turnover number (min⁻¹)</th>
<th>K₅₅</th>
<th>Effect of superoxide dismutase</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NMN acceptor</td>
<td>+FAD</td>
<td>-FAD</td>
<td>+FAD</td>
<td>-FAD</td>
</tr>
<tr>
<td>O₂</td>
<td>Native</td>
<td>0.25-2.5</td>
<td>0.25-1.5</td>
<td>0.4 mM</td>
<td>0.4 mM</td>
</tr>
<tr>
<td></td>
<td>Deflavine</td>
<td>0.25-2.5</td>
<td>0.25-1.5</td>
<td>0.4 mM</td>
<td>0.4 mM</td>
</tr>
<tr>
<td>Cyt c</td>
<td>Native</td>
<td>0.5-10</td>
<td>0.05-0.5</td>
<td>0.32 mM</td>
<td>0.32 mM</td>
</tr>
<tr>
<td></td>
<td>Deflavine</td>
<td>0.25-2.5</td>
<td>0.25-1.5</td>
<td>0.32 mM</td>
<td>0.32 mM</td>
</tr>
<tr>
<td>DCIP</td>
<td>Native</td>
<td>0.05-0.5</td>
<td>0.05-0.5</td>
<td>0.22 mM</td>
<td>0.22 mM</td>
</tr>
<tr>
<td>R²Fe(CN)₆</td>
<td>Native</td>
<td>0.05-0.5</td>
<td>0.05-0.5</td>
<td>0.22 mM</td>
<td>0.22 mM</td>
</tr>
<tr>
<td></td>
<td>Deflavine</td>
<td>0.05-0.5</td>
<td>0.05-0.5</td>
<td>0.22 mM</td>
<td>0.22 mM</td>
</tr>
<tr>
<td>NBT</td>
<td>Native</td>
<td>0.05-0.5</td>
<td>0.05-0.5</td>
<td>0.17 mM</td>
<td>0.17 mM</td>
</tr>
<tr>
<td></td>
<td>Deflavine</td>
<td>0.25-1</td>
<td>0.25-1</td>
<td>0.17 mM</td>
<td>0.17 mM</td>
</tr>
</tbody>
</table>

a Assay conditions as previously described (16).
b K₅₅ for NMN.
c At 10⁻⁶ M concentration.
d K₅₅ for Cyt c.
e K₅₅ for DCIP.
Concentration for native and deflavo enzymes, respectively. C, plot of the reciprocal turnover number versus reciprocal NMN concentration, taken from the intercepts of A and B. Native enzyme (○); deflavo enzyme (■). Conditions, air-equilibrated 0.05 M phosphate, pH 7.8, at 25°.

![Fig. 5](http://www.jbc.org/)

**Fig. 5.** NMN-ferricyanide reductase activity. A and B, plot of the reciprocal turnover number versus reciprocal NMN concentration for native and deflavo enzymes, respectively. C, plot of the reciprocal turnover number versus reciprocal NMN concentration, taken from the intercepts of A and B. Native enzyme (○); deflavo enzyme (■). Conditions, air-equilibrated 0.05 M phosphate, pH 7.8, at 25°.

Protein with a shoulder around 480 nm. Fig. 2 shows the spectra of the oxidized deflavo enzyme and that form reduced with NMN and the calculated difference spectrum having a peak at 470 nm.

**Kinetic Behavior of Native and Deflavo Aldehyde Oxidase in Respect to Some Electron Acceptors**

Previous reports (1, 17) described the effects of several inhibitors on the various catalytic activities of the enzyme and concluded that there were four sites of electron egress from the enzyme to groups of electron acceptors and suggested the presence in the enzyme of four carriers mediating electron transport from substrate to oxygen. With the preparation of a stable deflavo enzyme it was of obvious interest to test the different reductase activities of the enzyme in order to see what change was introduced into the system and which electron acceptors react with the flavin prosthetic group of the enzyme.

**Oxygen Reductase Activity—**When the NMN oxygen reductase activity was tested with the native enzyme at different oxygen concentrations no dependency on the electron acceptor concentration was found and a \( K_m \) for NMN of 0.4 mM was calculated (Fig. 3). The deflavo enzyme had no oxygen reductase activity but when preincubated at 25° for 3 min in presence of \( 1 \times 10^{-4} \) M FAD it recovered 73% of the original activity of the native enzyme.

**Cytocrome c Reductase Activity—**The absolute requirement for molecular oxygen in order to effect the reduction of Cyt. c was described previously (11). Fig. 4 shows the double reciprocal plot of the turnover number versus the Cyt. c concentration for native and deflavo enzymes, in the absence and presence of FAD. It should be noted that the turnover number for the native enzyme increases by a factor of 2 in the presence of FAD; this is probably due to reduction of Cyt. c by \( O_2^- \) produced via free FAD—accepting electrons from the reduced enzyme. Again the deflavo enzyme is devoid of any activity unless FAD is added. In the presence of FAD a 75% recovery of the activity of the native enzyme was found. Under those conditions turnover numbers of 56 and 39 were found, respectively, for native and deflavo enzymes with an identical \( K_m \) for NMN of 1.37 mM. As noted in Table II, this is the only activity of the enzyme which has been found to be inhibited by superoxide dismutase.

**Ferricyanide Reductase Activity—**This activity is not affected by removal of flavin from the enzyme. A turnover number of 230 has been found for both native and deflavo enzymes with a \( K_m \) for NMN of 0.32 mM. Fig. 5 also shows that the activity is not dependent on the concentration of the electron acceptor in the range tested while at concentrations higher than 0.5 mM a slight inhibition occurs.

**Dichlorophenolindophenol Reductase Activity—**This activity also is not affected by removal of the flavin from the enzyme. A turnover number of 230 has been found for both native and deflavo enzymes with a \( K_m \) for DCIP and NMN are calculated to be, respectively, 0.95 mM and 0.22 mM. These kinetic patterns appear to be in agreement with those found with the pig enzyme (18) indicating a ternary complex mechanism at least for the reduction of DCIP.

**Reduction of Nitro Blue Tetrazolium—**In contrast to a previous report (17) we find that under aerobic conditions the enzyme does not show any NBT reductase activity: such activity was observed only under strictly anaerobic conditions were employed. Fig. 7A is the plot of the reciprocal turnover number versus the reciprocal NBT concentration for native enzyme plus and minus added FAD. A turnover number of 74 and a \( K_m \) for NBT of 7.0 μM were calculated. The deflavo enzyme in the absence of added FAD is completely unable to reduce NBT.

Again, as for the reduction of Cyt. c, an increase in activity was found, even for the native enzyme, in the presence of added FAD. Fig. 7B shows the plot of the reciprocal turnover number versus the reciprocal NMN concentration in the presence of 1 \( \times 10^{-4} \) M FAD and 1 \( \times 10^{-4} \) M NBT. Under these conditions turnover num-
FIG. 7. NMN-NBT reductase activity. A, plot of the reciprocal turnover number versus reciprocal NBT concentration for native enzyme (○) and native enzyme in presence of 1 × 10⁻⁴ M FAD (□). B, plot of the reciprocal turnover number versus reciprocal NMN concentration for native (○) and deflavo (●) enzymes in presence of 1 × 10⁻⁴ M FAD. The activity was tested under anaerobic conditions, in 0.05 M phosphate, pH 7.8, at 25°C.

FIG. 8. The reduction of free FAD (1 × 10⁻⁴ M) by native (○) and deflavo (●) enzymes. Plot of the reciprocal turnover number versus reciprocal NMN concentration. The rate of reduction was independent of FAD concentration in the range tested (10⁻⁶ to 10⁻² M). Conditions, anaerobic in 0.05 M phosphate, pH 7.8, at 25°C.

Numbers of 285 and 145 were found for native and deflavo enzymes, respectively, with a Kₘ for NMN of 0.2 mM.

Reduction of Free FAD—As previously reported aldehyde oxidase is able to reduce free flavins under anaerobic conditions. Fig. 8 shows that such activity is dependent, in the concentration of FAD tested (10⁻⁶ to 10⁻² M), only on NMN concentration. A turnover number of 270 was found for the native enzyme and a Kₘ for NMN of 0.17 mM, while the reconstituted enzyme had a turnover number of 160 with the same Kₘ for NMN.

DISCUSSION

The modified preparation method described in this paper yields routinely reproducible results. The enzyme so obtained appears homogeneous by disc gel electrophoresis. The characteristic spectral ratio A₄₅₀/A₃₈₀ is 5.5, identical with the best value reported by Felsted and Chaykin (5) and considerably lower than that originally reported by Rajagopalan et al. (11). The NMN oxygen reductase activity, under the standard conditions of Felsted and Chaykin, is 2.0 to 2.2 i.u. per mg. This value is slightly higher than their best value of 1.89 i.u. per mg.

The properties of aldehyde oxidase are remarkably similar to those of xanthine oxidase, as has frequently been noted in the past (1, 2, and references therein). Both enzymes have similar broad substrate and acceptor specificity, the chief distinguishing feature being the inability of aldehyde oxidase to oxidize xanthine. Both enzymes have similar cofactor composition, containing 1 atom of molybdenum per eq of enzyme-bound FAD, and 4 atoms of nonheme iron associated with 4 atoms of acid-labile sulfur. As shown in the accompanying paper (4), the similarity has also been extended by the finding in aldehyde oxidase of an active site persulfide linkage, as had previously been described for xanthine oxidase (3, 19).

The similarity between aldehyde oxidase and xanthine oxidase is further emphasized by a comparison of their spectral properties. Based on the FAD composition, the extinction coefficient at 450 nm of native aldehyde oxidase is 34,700 M⁻¹ cm⁻¹. This should be compared to the value of 37,800 M⁻¹ cm⁻¹ reported for xanthine oxidase (13). It has also proved possible to remove the FAD component from aldehyde oxidase to yield a deflavo enzyme in a manner similar to that employed with xanthine oxidase (15). Again the spectral properties of the two enzymes are remarkably similar. As shown in Fig. 1, deflavo aldehyde oxidase has absorption maxima at 320, 425, and 460 nm, with an ε₄₅₀ value of 24,000 M⁻¹ cm⁻¹. Deflavo xanthine oxidase has absorption maxima at 320, 420, and 467 nm, with an ε₄₅₀ value of 25,300 M⁻¹ cm⁻¹ (15). The availability of an undenatured deflavo enzyme has permitted more definitive evidence to be obtained concerning the oxidation-reduction groups in the enzyme which are responsible for reaction with various electron acceptors. The NMN-oxygen reductase activity of the native enzyme is lost completely on removal of the flavin; 73% reconstitution of this activity can be regained on adding FAD. It would thus appear clear, as with xanthine oxidase (15, 19, 20), that the reduced flavin chromophore of the enzyme is the site of interaction with molecular oxygen. From the fact that the deflavo enzyme is also devoid of NMN-cytochrome c reductase activity, and that this activity of the native enzyme is completely inhibited by superoxide dismutase, and the previous demonstration that O₂⁻ is a good reductant of cytochrome c (21, 22), it may be concluded that it is the reduced flavin of the enzyme rather than the nonheme iron which is responsible for O₂⁻ production. Similarly it may be concluded that the flavin is the site of reaction with nitro blue tetrazolium. On the other hand the site(s) of interaction with other electron acceptors, such as ferricyanide and DCIP, must be the molybdenum or the iron-sulfur centers, since these activities are not affected by removal of flavin.

Studies with inhibitors such as amytal, oligomycin, and anti-
mycin A similar to those previously carried out with the native enzyme (1, 17), but now extended to the deflavo enzyme, should provide definitive answers to such questions.

REFERENCES