The Purification of a Nitro-reductase from Nocardia V*

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Although several nitro-reductase systems, either from animals or microorganisms, have been identified in intact cells or cell-free homogenates, partial purification of the enzyme has been reported only for extracts of Neurospora crassa (1) and Aspergillus niger (2, 3).

Purification of the enzyme or enzymes involved in the reduction of organic nitro compounds is important for two reasons. (a) There is doubt whether the activity can be attributed to a single enzyme or a combination of enzymes. (b) As Nason (4) has reported, it is unknown whether the nitro-reductase is a specific system or whether the nitro compounds can serve as nonspecific electron acceptors for a number of enzyme systems. A further point of interest arises from the suggestion that the reduction of inorganic nitro compounds might take place after their transformation or incorporation into an organic form (5). Although later studies with Neurospora mutants have not supported this hypothesis, the possibility that an organic pathway exists for nitrate reduction has not been completely discarded.

In order to study the properties of the purified nitro-reductase system and the mechanism of electron transfer, it was necessary to have a readily available source of crude material. Such a source is provided by a crude extract of Nocardia V.

The present paper describes a scheme for the separation and purification of the Nocardia nitro-reductase enzyme. Ammonium sulfate fractionation in conjunction with ion exchange chromatography provide a convenient and rapid procedure for the isolation of nitro-reductase in highly purified form. The enzyme preparation so obtained may still be contaminated with other substances.

EXPERIMENTAL PROCEDURE

Microbiological—Nocardia V used for these studies of nitroorganic compounds metabolism was grown in a glutamic acid-mineral salts medium described previously (6). For preparation of nitro-reductase, large quantities of cells were obtained by inoculating 2 liters of medium contained in a 5-liter flask. Satisfactory aeration of the cultures was obtained by stirring in the apparatus as described by Peel (7). The cells were harvested after growth for 28 hours at 30°C in a Sharples centrifuge and washed three times with 0.85% NaCl solution. The sedimented organisms were then resuspended in a small volume of 0.067 M phosphate buffer, pH 7.6, and added dropwise to 20 volumes of ice-cold acetone, which was stirred vigorously until coagulation occurred. The coagulated organisms were allowed to settle, and some of the clear supernatant solution was removed by decantation. The rest of the material was filtered on a Buchner funnel with moderate suction. The precipitate was washed with 100 ml of cold acetone followed by 100 ml of cold ether; gentle suction was maintained for a few minutes to dry the preparation, which was transferred to a vacuum desiccator over CaCl₂. All manipulations were carried out at room temperature. Powders prepared in this way retained their original activity for several months when preserved in a desiccator.

Materials were from the following sources: DPNH, Sigma Chemical Company; p-dinitrobenzene, ammonium sulfate (Analar grade), and protamine sulfate, The British Drug Houses, Ltd.; DEAE-cellulose, Brown Company; L-cysteine, Light and Company, Ltd. Calcium phosphate gel (about 35 mg per ml, dry weight) was prepared by the method of Keilin and Hartree (8).

Determination of activity for several months when preserved in a desiccator.

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Enzymatic—Nitro-reductase activity was assayed in terms of the p-nitroaniline formed from p-dinitrobenzene by the measurement of the reduction of p-dinitrobenzene by the measurement of the p-nitroaniline formed. The complete reaction mixture contained 1.8 x 10⁻⁴ M p-dinitrobenzene, 5 x 10⁻⁴ M L-cysteine, 1.08 x 10⁻² M DPNH, enzyme preparation, and 0.067 M phosphate buffer, pH 7.6, in a volume up to 2.5 ml. The reaction was usually started by the addition of DPNH after equilibration at 37°C. At the end of the incubation period (1 hour), 0.05 ml of 40% trichloroacetic acid (w/v) was added to each tube, the precipitate was removed by centrifugation and an aliquot of the supernatant was analyzed for arylamine by the standard procedure. Conditions were chosen in which the rate of formation of arylamine was proportional to the amount of enzyme present.

One unit of enzyme is defined as that amount of nitro-reductase that will form 1 μg of p-nitroaniline per hour at 37°C with DPNH as hydrogen donor under standard conditions. Specific activity is expressed as units per mg of protein.

RESULTS

Extraction—Extracts were prepared by exposing 5 ml of acetonitrile-powdered organisms (60 mg per ml) in 0.067 M phosphate buffer, pH 7.6, plus 1 mM cysteine to ultrasonic vibration of 20 kc per second generated by a 500-watt Mullard disintegrator. The cup was cooled by running tap water throughout the exposure. After exposure of 20 minutes to ultrasound, the suspen-
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DEAE-cellulose column eluate

Crude extract

Ammonium sulfate, 45-65% saturation

Purification of Nocardia nitro-reductase

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (ml)</th>
<th>Total units</th>
<th>Total protein (mg)</th>
<th>Specific activity (units/mg)</th>
<th>Enzyme yield (%)</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>40</td>
<td>3860</td>
<td>221.3</td>
<td>5.53</td>
<td>17</td>
<td>100</td>
</tr>
<tr>
<td>Ammonium sulfate, 45-65% saturation</td>
<td>40</td>
<td>1786</td>
<td>10.3</td>
<td>0.235</td>
<td>173</td>
<td>100</td>
</tr>
<tr>
<td>DEAE-cellulose column eluate</td>
<td>8</td>
<td>1393</td>
<td>0.36</td>
<td>0.045</td>
<td>3876</td>
<td>35</td>
</tr>
</tbody>
</table>

**Fig. 1.** Chromatography of partially purified nitro-reductase (specific activity, 170) on DEAE-cellulose ion exchange agent, in a column 1 X 18 cm. Removal of protein and enzyme by gradient elution in increasing NaCl concentration in the presence of phosphate buffer, pH 7.6, containing 1 mM cysteine. Salt gradient from 0 to 0.4 M NaCl. Other details are given in the text.

A solution was diluted to 40 ml with cold phosphate buffer and centrifuged for 15 minutes at 4000 x g in a MSE centrifuge in the cold room. The supernatant extract contained the enzyme and was used as starting material for further purification. All steps were carried out at 4°C.

**Ammonium Sulfate Fractionation**—To 40 ml of the crude extract, 0.273 g of solid ammonium sulfate per ml was added until 45% saturation was obtained. The mixture was stirred for 15 minutes, and the pH was kept near 7.6 by careful addition of 0.5 to 2.0 ml of 0.01 M NaOH. After centrifugation, the precipitate was discarded and 0.133 g of ammonium sulfate per ml was added to 65% saturation to the supernatant. After centrifugation, the supernatant solution was discarded, and the active precipitate was dissolved in cold phosphate buffer, pH 6.5. The specific activity of this fraction was 173. Table I gives a summary of the purification procedure.

**DEAE-cellulose Chromatography**—To minimize possible denaturation of protein, all chromatographic experiments were carried out in a cold room at 4°C; 1 mM cysteine was added to all buffers as protective agent.

Starting material for all these experiments was obtained by ammonium sulfate fractionation of Nocardia extracts as described above; the fraction of specific activity 170 to 180 was used and dialyzed against the same buffer as that used in the preparation of the column.

The preliminary experiments had indicated that adsorption of nitro-reductase on DEAE-cellulose columns takes place at a pH value greater than 7.0. Columns of DEAE-cellulose, 1 x 18 cm, buffered with 0.01 M phosphate buffer, pH 7.6, were prepared, and the dialyzed enzyme fraction was applied. No protein was eluted by subsequent passage through the column of 5-column volumes of the buffer. The effect of washing the column with salt solutions of increasing ionic strength was then studied; the pH was maintained at 7.6 in 0.01 M phosphate buffer, and the ionic strength was increased by the addition of graded amounts of NaCl. The salt gradient was obtained by means of two reservoirs; one contained 150 ml of the 0.01 M phosphate buffer and the other contained 150 ml of 0.4 M NaCl. After 4-ml fractions were collected, elution of protein took place, and the enzyme was obtained as a sharp peak leaving the column after a high proportion of the inactive protein had been eluted. The pattern of the chromatography is described in Fig. 1. The elution of protein was followed spectrophotometrically by determining the absorbance at 280 nm. Enzyme activity determinations were made on each fraction by the standard assay system.

The data reported in Table I for the DEAE step were obtained from the pooled fractions showing the highest specific activity. Since the absorbance readings were low, the protein determination and, consequently, the specific activity for the fractions of the DEAE step have only an indicative value. The recovery of protein varied between 80 and 90%. Complete recovery could be obtained by final elution of the column with 0.1 M carbonate-bicarbonate buffer, pH 9.2. The recovery of activity represented a yield of 66%. The increase in specific activity represented a purification of 228 times over that of the crude enzyme preparation. A longer column (1 x 30 cm) gave no better resolution of the enzyme. The recovery values indicate considerable loss of activity, suggesting that the enzyme stability is affected by either the removal of cofactors or protective impurities. No explanation of these losses has been found; reactivation is not obtained by the addition of any of the flavins.

The result in Fig. 1, where the highest activity corresponds to the lowest protein content, is not the expected result for elution of a homogeneous protein. Attempts at rechromatography on DEAE-cellulose were unsuccessful, either for establishing homogeneity or for improving the purity of the preparation.

**Ultracentrifugal Analysis**—The highly purified nitro-reductase fraction (specific activity, 3700) from the DEAE-cellulose column was examined in a Spinco model E ultracentrifuge. The material from a large number of separate preparations was combined in order to get enough for the ultracentrifuge run. The effluent of the tubes from several DEAE-cellulose column chromatographic runs containing the enzyme were pooled together, and solid ammonium sulfate was added slowly with gentle stirring to achieve a final concentration of 70%. When the addition of the salt was completed, the suspension was kept for 8 to 10 hours in the cold and then centrifuged. The supernatant was discarded, and the precipitate was suspended in as small a volume as possible of 0.1 M phosphate buffer, pH 7.6, containing cysteine. The suspension was centrifuged at 4°C at 20,000 x g for 20 minutes, and any small precipitate formed was discarded. Determinations in the ultracentrifuge were carried out at 22°C in 0.11 M phosphate buffer, pH 7.6. Some of the patterns obtained are
shown in Fig. 2. A single, slowly moving component was observed, which sedimented as a broad peak with $s_{20,w} = 2.18$ S. There was no indication of more rapidly sedimenting material. In another ultracentrifuge run by using the method of Archibald (12), and by assuming a partial specific volume of 0.73 ml per g, the molecular weight was estimated to be 24,000. This value represents an approximate molecular weight only, since the examination was carried out only at a single dilution, the value for the partial specific volume being assumed and not determined experimentally.

**DISCUSSION**

Fractionation on DEAE-cellulose ion exchange agent gave rise to highly purified preparations of the enzyme although recoveries showed considerable loss of activity as the purity increased, indicating that enzyme stability is affected by the removal of impurities. However, no experimental data, so far, have been obtained to explain the nature of these losses. Other purification procedures tested gave less satisfactory results.

Separation under the conditions reported yielded nitro-reductase in a highly purified state, and the data presented above favor the view that the enzyme has been isolated in homogeneous form. However, the degree of purity of the *Nocardia* nitro-reductase preparation obtained has not been fully determined since insufficient material was available for detailed physical characterization. The purity has been judged mainly by the behavior of the preparation on the ultracentrifuge; the nitro-reductase preparation behaved as a homogeneous protein in so far as characterization by sedimentation procedures will permit such a description. Whereas no specific claim of purity can be made on the basis of such incomplete data, the high specific activity and freedom from other activities that are present in readily measureable amounts in the crude extracts, e.g. xanthine oxidase (13, 14), suggest that if the nitro-reductase activity involves more than one enzyme, then these enzymes must possess very similar properties.

No satisfactory explanation can be put forward for the observation that nitro-reductase undergoes considerable inactivation in the course of purification. Inactivation of the enzyme might be caused by denaturation of the protein or by dissociation of a prosthetic group. Inactivation of partially purified preparations of nitro-reductase can be prevented to some extent by addition of L-cysteine. The highly purified enzyme preparation is very labile and although L-cysteine effects some protection, the only method that can be used to keep the enzyme in an active form is to keep the preparation frozen.

Although it would be interesting to compare the physical properties of the *Nocardia* nitro-reductase with those of other nitro-reductases isolated from other sources, such a comparison is not yet possible since the relevant data for other enzymes are not available.

It is not yet clear whether all the steps in the sequence involved in the reduction of nitro compounds are catalyzed by a single enzyme or require a complex of enzymes. Zucker and Nason (1) have shown that Neurospora crassa extracts possess a system that can reduce $p$-dinitrobenzene either to $m$-nitrophenylhydroxylamine or $m$-nitroaniline but no other evidence has been reported so far. The data reported in this communication provide a good indication that the nitro-reductase from *Nocardia* V consists of a single component which is able to carry out the complete reduction of aromatic nitro groups to the corresponding amino groups. The ultracentrifuge results would suggest that, if there are a number of specific enzyme-catalyzed steps, then the enzymes concerned must possess very similar properties. This is further emphasized by the fact that under no conditions has the nitro-reductase activity of the "65% fraction" been obtained in more than one fraction, so that it has not been possible to demonstrate more than one active protein by means of fractionation techniques.

If it is assumed that three different steps are involved in $p$-dinitrobenzene reduction by *Nocardia* nitro-reductase and that the enzyme system consists only of one protein, then the enzyme may show multifunctional and catalytic activities as has been reported for some systems by Rutter and Lardy (15) and has been shown by various workers for more versatile enzymes (16-18).

A different explanation would be that it is only the first step of the reduction which is carried out by enzymatic action, the other steps taking place by purely chemical processes. Saz and Slie (19) have reported that $p$-nitrosobenzoic acid is reduced to a diazotizable compound in the presence of L-cysteine, and if the same happens with a possible nitroso compound intermediate in the $p$-dinitrobenzene reduction process, then no accumulation of intermediates could be demonstrated. However, this suggestion is not supported by the experimental results, since one would then expect accumulation of intermediate compounds in the absence of added sulfhydryl compound. After protein denaturation, addition of the sulfhydryl compound would result in the transformation of such intermediates into diazotizable material. Since this does not occur, it would appear that the last steps of the reduction process are not spontaneous but are under some form of control.

Insufficient material on the comparative aspects of nitro reduction is available to justify any broad generalizations. It will be of considerable interest to extend comparative studies, since relatively little work has been done on enzymes from different sources; in particular, the possible differences in coenzyme requirements need investigation.

**SUMMARY**

A nitro-reductase system has been extracted from *Nocardia* V. Highly purified preparations (228 times) have been obtained by
the use of diethylamino ethyl cellulose after ammonium sulfate fractionation. Ultracentrifugal analysis of those preparations shows a single component corresponding to a protein of low molecular weight (24,000).

From the behavior of the enzyme system on fractionation and ultracentrifugal analysis, the conclusion is drawn that the reduction of nitro groups to amino groups is carried out by one enzyme or, if more than one enzyme is concerned, then these enzymes must have very similar properties.

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