Purification and Properties of a Disulfide Reductase Obtained from *Achromobacter starkeyi*

J. Ruiz Herrera, R. Amezquía Ortega, and A. Trujillo

From the Departamento de Microbiología, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, México, D. F.

**SUMMARY**

A disulfide reductase with characteristics different from those previously described in the literature was isolated from *Achromobacter starkeyi*. The enzyme was purified approximately 90-fold from cell-free extracts prepared with a Hughes press. Oxidized glutathione was the only natural substrate utilized by the partially purified enzyme. However, the enzyme showed a higher affinity and higher activity for the synthetic compound 5,5'-dithiobis(2-nitrobenzoic acid). The enzyme could utilize both NADH and NADPH as electron donors, but it was more active with NADPH. The optimum pH was 7.0, and the optimum temperature was about 28°C. The enzyme was highly susceptible to the presence of divalent cations and iodacetate, but arsenite and N-ethylmaleimide showed only a slight inhibitory effect. The enzyme was slowly inactivated when it was incubated in the presence of NADH or NADPH.

Recently we described the presence of a peculiar disulfide reductase in a bacterium isolated from soil on a medium containing methionine as the sole source of carbon, nitrogen, and sulfur (1). This disulfide reductase did not appear to be involved in methionine metabolism and seemed to be very specific with only 5,5'-dithiobis(2-nitrobenzoic acid) as a substrate. All other disulfides tested were not substrates of the crude cell-free extract.

This enzyme was shown to be similar to the enzyme described by Black et al. (2), but we could not reach a final conclusion about this because crude extracts had a high NADH dehydrogenase activity which obscured the results.

The purification of the enzyme has been undertaken in order to investigate its properties further. The properties of the purified enzyme preparation are the subject of the present communication.

**EXPERIMENTAL PROCEDURE**

**Chemicals**—5,5'-Dithiobis(2-nitrobenzoic acid), lipoic acid, dimethyl disulfide, and N-ethylmaleimide were obtained from K and K Laboratories. NADH (92.6% purity), NADPH, DEAE-cellulose, carboxymethyl cellulose, cytochrome c, mercaptoethanol, L-methionine-2S-sulfoxide, DL-methionine, sodium iodacetate, P-chloromercuribenzoate, and yeast glutathione reductase (NAD(P)H-glutathione oxidoreductase, EC 1.6.4.2) were purchased from Calbiochem. Cystathionine, cystine, GSSG, calcium phosphate gel, alumina C gel, and protamine sulfate were obtained from Nutritional Biochemicals. 2-Keto-4-methyl-mercaptobutyric acid was obtained from Bios Laboratories, purified starch from Buchler Instruments, and EDTA from Eastman. Hydroxyethyl disulfide was prepared from mercaptoethanol as described by Black (3).

**Strain**—The bacterium used in this study was isolated from a soil in a medium containing methionine as the sole source of carbon, nitrogen, and sulfur. It was maintained on slants of a synthetic Medium M previously described (1). It was identified as an *Achromobacter* and designated tentatively as *Achromobacter starkeyi* nova sp. Its general properties will be described elsewhere.

**Cultivation of Bacterium and Preparation of Cell-free Extracts**—Large batches of cells were cultivated for 16 hours in Trypticase broth (Baltimore Biological Laboratory, Baltimore, Maryland) by shaking 200 ml of medium in 1000-ml Erlenmeyer flasks at 28°C.

Cells were harvested with a Sharples supercentrifuge. The cells obtained from about 10 liters of medium were washed three times with 0.05 M phosphate buffer, pH 7.3, containing 1 mM EDTA, by centrifugation in an International HR-1 refrigerated centrifuge. The cells (60 to 80 g, wet weight) were finally resuspended in about 50 ml of the same buffer and disrupted in a Hughes press. A pressure of 20,000 psi was applied with a Carver Laboratory press. The extract thus obtained was highly viscous and impossible to centrifuge. It was therefore submitted to sonic treatment in a Raytheon sonic oscillator for periods of 5 min. The resulting material was centrifuged at 15,000 × g for 15 min. The supernatant or crude extract was the initial material used for the purification process described below. During the preparation of cell-free extracts and all successive steps the temperature was maintained below 2°C.

**Enzyme Assays**—Disulfide reductase activity was measured spectrophotometrically by following the formation of the yellow sulfide formed in reduction of 5,5'-dithiobis(2-nitrobenzoic acid)
at 412 nm (4). One unit of enzyme is defined as the amount which catalyzes the formation of 1 μmole of sulfide per min. Specific activity is expressed as the number of units per mg. The millimolar extinction coefficient of the sulfide was that given by Flavin (5).

In some experiments NADH and NADPH oxidation was measured at 340 nm. Micromoles of NADH oxidized were calculated with a millimolar extinction coefficient of 6.22 (6). Two micromoles of sulfide are formed per μmole of NADH or NADPH utilized.

Initial rates of activity were measured with a Beckman DU spectrophotometer connected to a Beckman Sera attachment to a Photovolt Varicord model 43 recorder. All assays were conducted at 28° in 3-ml cells of 1-cm light path.

The routine assay mixture, unless otherwise stated, contained in a final volume of 3 ml: 0.05 M potassium phosphate, 1 mM NADH, or NADPH, 66.6 μM DTNB, and enzyme at pH 7.3. The reaction was started by the addition of NADH or NADPH.

NADH dehydrogenase was measured spectrophotometrically as described above for disulfide reductase by following the decrease in absorbance at 340 nm. The incubation mixture contained 0.05 M potassium phosphate, 0.1 mM NADH, and enzyme at pH 7.3. The reaction was started by the addition of the substrate.

NADH oxidation with other electron acceptors was measured as described for disulfide reductase by following the decrease in absorbance at 340 nm. Cytochrome c, 10 μM, 66.6 μM DPI, or 66.6 μM potassium ferriyanide was added instead of DTNB.

Electrophoresis of Enzyme—Starch blocks of 20 × 3 × 1.5 cm were prepared and the enzyme was added at a point 4 cm from the cathode. The enzyme was subjected to electrophoresis with a constant current of 20 ma for 19 hours in 0.085 M Veronal buffer, pH 8.6. The block was cut in 1-cm sections and the protein was eluted by shaking with 5 ml of 0.05 M phosphate buffer, pH 7.3, containing 1 mM EDTA, for 15 min. Starch was removed by centrifugation and the extraction was repeated. Both electrophoresis and extraction were carried out at 4°.

Electrophoresis on acrylamide gel was carried out by standard techniques (7). Bands of active fractions were located by incubating the gels with the standard incubation mixture and precipitated material was dissolved in 63 ml of 0.05 M phosphate buffer, pH 7.3. The gel was poured on a carboxymethyl cellulose column of 1 × 10 cm and the enzyme was eluted with 0.05 M phosphate buffer, pH 7.3, containing 1 mM EDTA. Fractions of 7.5 ml were collected, with a flow rate of 0.5 ml per min. All of the enzyme appeared in Fractions 2 and 3. Table I summarizes the results obtained with this method of purification. With this procedure NADH dehydrogenase was eliminated during DEAE-cellulose chromatography.

Properties of Purified Enzyme

Stability—The enzyme was very stable at -20°. When kept at this temperature it remained fully active within any appreciable loss of activity for periods of 2 to 3 months.

Effect of pH—The purified enzyme had an optimal pH of about 7.0. Activity decreased sharply at lower and higher pH values.

<table>
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<th>Stage</th>
<th>Volume</th>
<th>Total protein</th>
<th>Total enzyme</th>
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1 The abbreviations used are: DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); DPI, 2,6-dichlorodinophenol; NEM, N-ethylmaleimide; CMB, p-chloromercuribenzoate.
Effect of Temperature—Enzyme activity was maximal at 28°C. Above 45°C the enzyme appeared to be irreversibly denatured (Fig. 1).

Effect of Enzyme Concentration—Fig. 2 shows that the reaction rate was linearly dependent on the amount of enzyme added, up to 0.014 unit.

Specificity of Enzyme Concentration—The enzyme was highly active with the synthetic substrate DTNB. The only other disulfide reduced was GSSG but enzyme activity with this substrate was very low, about one-eighth of that with DTNB. The enzyme did not reduce the following disulfides: cysteine, homocysteine, hydroxyethyl disulfide, methyl disulfide, and lipoic acid, nor were L-methionine-dl-sulfoxide or the thiocysteine, 2-thiochrome, cystathionine, and 2-keto-4-methylmercaptobutyric acid, reduced by the enzyme. Each substrate was tested at three different concentrations: 66.6 μM, 0.666 mM, and 1.666 mM. When purified cell walls of the bacterium were used as substrate, no NADH oxidation could be detected.

Albumin (2.41 mg in a final volume of 1.1 ml) was tested as substrate, measuring the activity by titration of −SH groups with CMBS. No increase in −SH groups could be detected at the end of an incubation period of 1 hour. If the enzyme would be a protein disulfide reductase it has to be concluded that it must be very specific.

The disulfide reductase was active with both NADH and NADPH (Fig. 3). According to the specificity of the electron donor, this enzyme is different from yeast glutathione reductase which utilizes only NADPH as electron donor, as is shown in the same figure. In addition, yeast glutathione reductase is unable to utilize DTNB as substrate, at least at a concentration which saturates the disulfide reductase described in this paper, as shown in Fig. 1.

The reasons that we believe that a single enzyme is responsible for the reduction of GSSG and DTNB, and utilizes both NADH and NADPH as electron donors, are the following. (a) The ratio of both activities (DTNB:GSSG) remained nearly constant during the last steps of purification, between 8 and 9 for a substrate concentration of 66.6 μM which was saturating for DTNB (activity of GSSG reduction could not be measured while there was NADH oxidase contaminating the preparation). (b) The enzyme was more active for a given substrate concentration with both substrates when NADPH was used as electron donor, but the enzyme had a higher affinity for both substrates when NADH was used as electron donor, as is shown below. (c) All of the activities were decreased to the same extent when the
Disulfide reductase with NADPH
GSSG reductase with NADPH
GSSG reductase with NADH

Fig. 4. Substrate specificity of GSSG reductase and disulfide reductase. Aliquots of 0.05 ml of disulfide reductase and GSSG reductase (1 mg per ml) were incubated with 0.8 μmole of DTNB and 0.3 μmole of either NADH or NADPH, and absorbance at 412 nm was recorded. No activity was observed with GSSG reductase. At arrows, 2.5 μmoles of GSSG were added to the indicated samples. Appearance of yellow color in this case is due to thiolysis of DTNB by free sulfhydryl groups. Curves were taken from direct tracings of the recorder. Other conditions were as in Fig. 1.

Fig. 5. Effect of DTNB concentration on disulfide reductase activity. The assay conditions were the same as described under “Experimental Procedure” and in the legend for Fig. 1. Activity is expressed as micromoles of sulfide formed per min per mg of protein.

Fig. 6. Effect of GSSG concentration on disulfide reductase activity. The assay conditions were as follows: 0.05 ml of disulfide reductase (0.20 unit per ml) and 0.15 ml of either 2 mM NADH or 2 mM NADPH, all dissolved in 0.05 M KH2PO4 buffer, pH 7.3, with EDTA, were incubated with variable amounts of 5 mM GSSG solution, and the change in absorbance at 340 nm was recorded. Activity is expressed as micromoles of GSH formed per min per mg of protein.

Fig. 7. Effect of NADH concentration on disulfide reductase activity. The assay conditions for activity with DTNB were the same as described under “Experimental Procedure” and in the legend for Fig. 1. The assay conditions for measurement with GSSG were similar to those described in Fig. 7 except that a constant volume of 0.2 ml of 20 mM GSSG was used. Activity is expressed as micromoles of sulfide formed per min per mg of protein.
With the partially purified enzyme preparation, a slight NADH oxidation could be detected when DPI or potassium ferricyanide was used as electron acceptor. Cytochrome c was inactive as acceptor. However, this activity could be separated from disulfide reductase activity by electrophoresis in a starch block.

Effect of Several Cations on Activity—As previously reported for the crude enzyme (1), purified enzyme was highly susceptible to divalent cations, the most toxic being Hg++ and the least toxic Mn++ (Table II).

Effect of Some Inhibitors—Table III shows the results obtained when other inhibitors were added to the reaction mixture. Chloride ion, which is an inhibitor of yeast glutathione reductase (10), had no action on the enzyme. On the other hand, 0.05 M sodium azide, which is also inhibitory for yeast glutathione reductase (10), inhibited the enzyme markedly. Iodoacetate, 1 mM, completely inhibited the enzyme, but 1 mM arsenite had only a slight effect.

NEM, 1 mM, also strongly inhibited the enzyme. Colman and Black (11) observed that N-ethylmaleimide inhibited glutathione reductase only when a particular -SH group associated with FAD was made accessible to NEM by incubation with NADPH+. We did a similar experiment, incubating the enzyme for 30 min with NEM either alone or in the presence of NADH, the controls being incubated with NEM alone. The enzyme was inhibited completely by incubation with NADH in the absence of NEM (Table III).

In further experiments the enzyme was incubated with either NADH or NADPH, and the kinetics of decrease of activity was followed. It was observed that the enzyme was inactivated most when it was incubated with NADH. When the incubation was carried out in the absence of nucleotides, activity was not significantly affected. These results are shown in Fig. 8.

Enzyme inactivated by incubation with NADH was inactive when tested with NADPH as electron donor, and also when GSSG was used as substrate. This result is taken as further evidence that a single enzyme is responsible for all of the activities.

DISCUSSION

The most peculiar characteristic of this enzyme is its affinity for a synthetic, not naturally occurring substrate. It may be said that this enzyme is a glutathione reductase having a higher affinity for DTNB. However, there are several differences between it and the glutathione reductases previously described, the main one being that this enzyme is active with both NADH and NADPH. In contrast, known glutathione reductases use
only NADPH (10, 12; see also Fig. 4), and yeast glutathione reductase cannot utilize DTNB as substrate (Fig. 5). In addition, the sensitivities of the two enzymes to several inhibitors are different.

Several enzymes have been described in the literature which can reduce several disulfides and all use NADPH as electron donor. Black et al. (2) described a yeast enzyme which required three fractions for reduction of methionine sulfoxide. Two of these three fractions were required for the reduction of several disulfides, one of which was DTNB. This enzyme differs from the one described here because of the difference of electron donor, the requirement for two protein fractions for activity, and the reduction of several disulfides in addition to DTNB.

Bandurski, Wilson, and Asahi (13-15) described a disulfide reductase involved in sulfate reduction. This enzyme is similar to the disulfide reductase described in this paper in that the only alternative substrate was glutathione. However, there are several differences. That enzyme was not affected by Hg++, it utilized NADPH but not NADH, and 0.5 mM arsenite was highly inhibitory.

In addition, this enzyme could use dyes as electron acceptors, whereas such activity could be separated from the disulfide reductase described in this paper. In this respect this enzyme is also different from lipoic dehydrogenase, which has “diaphorase” activity (16).

An enzyme involved in the conversion of CDP to deoxy-CDP, similar to Black’s disulfide-reductase, has been described (17). This enzyme reduces DTNB only in the presence of catalytic amounts of its natural substrate, thioredoxin. This enzyme resembles the system involved in the oxidative decarboxylation of glycine from Peptococcus glycinophilus which also reduces DTNB through a low molecular weight heat-stable protein which contains a functional disulfide (18).

Regarding the physiological role of the enzyme described in this paper it is difficult at the moment to arrive at any conclusion, but it may be assumed, as already mentioned, that it is a GSSG reductase having a higher affinity for the unnatural substrate DTNB and with other characteristics different from those of the GSSG reductases of other organisms.

REFERENCES

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