Preliminary Crystallographic Data for Glycolate Oxidase from Spinach*

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Glycolate oxidase, an enzyme that plays an important role in photosynthesis in plants, has been purified from spinach and crystallized in two different crystal forms. Form A which was obtained with tertiary butanol as precipitating agent belongs to space group I 422 with unit cell dimensions \(a = b = 148.1\) Å and \(c = 154.9\) Å. This form diffracts to high resolution and will be used for further crystallographic studies. Form B is also tetragonal, space group I42212, with cell dimensions \(a = b = 148.1\) Å and \(c = 104.2\) Å. This form was obtained from ammonium sulfate precipitations. Sodium dodecyl sulfate polyacrylamide gel electrophoresis shows that the enzyme is built up from subunits of molecular weight 37,000. The asymmetric units of both crystal forms contain at least two such subunits.

One possible way to increase plant productivity significantly might be to decrease the photosynthetic process in plants that have C-3 metabolism (1, 2). During this process reduced carbon atoms are withdrawn from the Calvin cycle into the glycolate pathway (3) and there are reoxidized to CO₂. The primary reaction of photosynthesis is the production of glycolate from ribulose 1,5-diphosphate (RuDP) and molecular oxygen (4). This reaction involves a factor, possibly a metalloenzyme which can be separated from the CO₂ fixation enzyme, ribulose 1,5-diphosphate carboxylase (5, 6). The first step in the glycolate pathway is the oxidation of glycolate to glyoxylate, catalyzed by the FMN-containing enzyme glycolate oxidase (glycolate:oxygen oxidoreductase EC 1.1.3.1). It has been demonstrated that inhibition of this enzyme decreases photosynthesis significantly (7). As part of a study to utilize the molecular structures of some of the enzymes involved in photosynthesis for rational design of specific inhibitors of this process, we now report crystallization and preliminary crystallographic data on glycolate oxidase from spinach.

**Purification and Crystallization**

The spinach used in this work was grown under controlled conditions in a greenhouse and harvested after about 7 weeks. The enzyme is purified using the following modifications of the procedure for the pea enzyme by Kerr and Groves (8). The spinach enzyme was collected in a different interval of ammonium sulfate precipitation, between 12 and 30% saturation. After proteolysis, the crude enzyme preparation was added to a solution of ammonium sulfate to a concentration of 20 mg/ml in 0.05 M Tris/HCl buffer, pH 8.3. The crystals obtained in this way are large, yellow, and polyhedral.

Two different crystal modifications have been obtained. Form A was obtained by the Zeppezauer microdialysis method (9) using a protein concentration of 20 mg/ml in 0.05 M Tris/HCl buffer, pH 8.3. The outer solution contained 50% v/v of tertiary butanol as precipitating agent and 0.25 me/ml of FMN in 0.05 M Tris/HCl buffer, pH 8.3. The crystals obtained in this way are large, yellow, and polyhedral.

Form B was obtained by the hanging drop method (10). Each drop, 20 µl, contained 20 mg/ml of the enzyme and ammonium sulfate to 10% saturation in 0.05 M Tris/HCl buffer, pH 8.3. The drops were equilibrated with higher ammonium sulfate concentrations in the same buffer. In a series of different experiments the concentration was varied from 12 to 20% saturation. Each experiment gave crystals which were shaped like cubes or parallelepipeds.

**UNIT CELL CONTENT AND ESTIMATION OF SUBUNIT MOLECULAR WEIGHT**

Crystals were mounted in thin walled glass capillaries in the usual way and diffraction patterns of the three different centric projections were recorded with a Buerger precession camera. Fig. 1 shows thehk0-projection of Form A crystals using nickel-filtered CuKα radiation from an Elliott rotating anode generator, crystal-to-film distance of 7.5 cm.

Form A crystals are tetragonal with unit cell dimensions \(a = b = 148.1\) Å and \(c = 134.9\) Å. The only unique extinction conditions present are that reflections where \(h + k + l = 2n + 1\) are absent. The space group is thus I422 with 16n subunits/unit cell, where \(n\) is the number of subunits per asymmetric unit.

Form B crystals are also tetragonal with unit cell dimensions \(a = b = 145.4\) Å and \(c = 104.2\) Å. The extinction conditions are that reflections h00 and k00 are present only for \(h = 2n\) and 00l are present only for \(l = 2n\). The space group is thus P42212 with 8n subunits/unit cell.

The subunit size of this enzyme was not previously known. Frigerio and Harbury (11) deduced from ultracentrifugation studies that the spinach enzyme exists as an equilibrium between two states with molecular weights 140,000 and 270,000. Corresponding values for the enzyme from pea leaves have been reported (8) as 50,000 and 100,000. Approximate calculations based on absorbance measurements were also made for the spinach enzyme (11) of the minimal molecular
weight that bound 1 FMN molecule. A value of 70,000 was obtained.

In order to obtain a reliable estimate of the molecular weight of the subunit we made a sodium dodecyl sulfate polyacrylamide gel electrophoresis. (We are grateful to Dr. B. Nordström, of this Institute, who carried out this work). A modification of the procedure by O’Farrell (12) was used with the following marker proteins: RNA polymerase from Escherichia coli (Mr = 165,000, 155,000, and 39,000) serum albumin (Mr = 68,000) and trypsin inhibitor (Mr = 21,500).

This procedure showed one protein band which by comparison with the bands of the series of known proteins corresponds to a molecular weight of 37,000. There are no bands with molecular weight larger than 37,000.

We can now use this value of 37,000 to estimate how many such subunits that could possibly occur per asymmetric unit in our crystals. This is most conveniently done by calculating the packing density, $V_n$, for different values of $n$ (13). The range of $V_n$ normally encountered for crystalline proteins are 1.68 to 3.53 Å³/dalton (13). For Form A crystals these values are 5.00 for $n = 1$, 2.50 for $n = 2$, and 1.67 for $n = 3$. Thus only for two such subunits does the value of $V_n$ fall within this range. We thus conclude that the asymmetric unit of these crystals in all probability contains two subunits of molecular weight 37,000.

For Form B crystals the values of $V_n$ are 3.7 for $n = 2$, 2.5 for $n = 3$, and 1.9 for $n = 4$. From these values we can only deduce that the asymmetric unit must contain at least two subunits.

Form A crystals are easy to obtain, contain FMN, and diffract to high resolution. Work is now in progress to obtain heavy atom derivatives of this crystal form for a high resolution structural study.

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