The X-ray Structure of Yeast Inorganic Pyrophosphatase

CRYSTAL PROPERTIES*

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SUMMARY

Single crystals of the yeast enzyme inorganic pyrophosphatase that are suitable for x-ray analysis have been grown. The space group of the crystals is P21. Their unit cell dimensions are: a = 70.4, b = 95.4, c = 52.2 Å, and β = 99.8°. There is 1 dimeric enzyme molecule with a molecular weight of approximately 68,000 in the asymmetric unit of the unit cell. The crystal is approximately 57% protein by weight.

Inorganic pyrophosphatase (EC 3.6.1.1 pyrophosphate phosphohydrolase) catalyzes the hydrolysis of inorganic pyrophosphate to orthophosphate. The apparent metabolic function of this species of enzyme, which is ubiquitous throughout nature, is to dispose of the inorganic pyrophosphate generated in various biosynthetic reactions. These reactions typically have equilibrium constants on the order of unity. Therefore the hydrolysis of pyrophosphate, which has a standard Gibbs free energy of ΔG° = -8.0 Cal per mole, drives these biosynthetic reactions essentially to completion (2).

Inorganic pyrophosphatase from baker's yeast (Saccharomyces cerevisiae) was first purified and characterized by Kunitz (3). Its properties have been recently reviewed by Butler (4). This enzyme, as do most phosphorlytransfer enzymes, requires divalent cations such as Mg2+ for catalytic activity. Its molecular weight, which has been measured using several techniques, has been reported to be in the range 63,000 to 71,000 (5-7). There has been some controversy in the literature concerning the subunit structure of the yeast enzyme. It was reported by Negi et al. (6, 8) that the enzyme consists of a single polypeptide chain. However, Avaeva et al. (9) observed that sodium dodecyl sulfate caused the enzyme to dissociate into two subunits, each with a molecular weight of 35,000. Gel electrophoresis of the enzyme in 8 M urea yielded a single band, suggesting that the subunits are identical (7). This has been corroborated by the studies of Heinrikson et al. (10) who found no evidence of multiple amino acids at any position of the 1st 20 NH2 terminal amino acid residues in the enzyme. Quantitation of this analysis is in accord with the hypothesis that the enzyme consists of two identical polypeptide chains. The sequence analysis of the 1st 5 COOH-terminal amino acid residues of the enzyme is in agreement with the foregoing conclusion (10).

Avaeva et al. (11) have reported that yeast inorganic pyrophosphatase is a tetramer of the type α2β2 in which the subunits have molecular weights of 14,000 and 17,000. However, recent results reported by Eifler et al. (12) strongly suggest that this discrepancy is an artifact due to limited proteolysis of the enzyme during its preparation. This also accounts for disagreements in the literature as to the COOH-terminal amino acid sequences of the enzyme (6, 10, 13). Of course one cannot completely rule out the possibility that the enzymes studied by the various groups were isolated from differing strains of baker's yeast.

Inorganic pyrophosphatase was isolated and purified as has been described by Cooperman et al. (14). The enzyme, which had been stored at a temperature of −20° as ammonium sulfate-precipitated pads, was dissolved in deionized water, desalted on a Sephadex G-25 column, and lyophilized. All subsequent work on the enzyme, including the x-ray measurements, was performed in a cold room at 4°. Lyophilized enzyme was dissolved in an aqueous solution that was 10% by volume in freshly redistilled 2-methyl-2,4-pentandiol (MPD) and made to 0.03 M in 2-(N-morpholino)ethanesulfonic acid (MES) buffer by the addition of a 0.5 M stock solution of MES1 that had been adjusted to pH 6.0 with NaOH. The enzyme concentration in this solution was 10 μg per ml. After several days a white, enzymatically inactive precipitate appeared in the solution in amounts that varied from preparation to preparation. This was removed by centrifugation to yield the solution of the enzyme.

The enzyme was crystallized by the method of vapor diffusion. From 0.020 to 0.030 ml of the enzyme stock solution was deposited on a microscope well slide that had been previously cleaned, soaked in the above MES stock solution, siliconized in a 1% aqueous solution of Siliclad (Clay-Adams), and air-dried. The slide was then sealed in a plastic box over a reservoir containing 10 ml of an aqueous solution that was 0.03 M in the MES buffer and 16% MPD by volume. The resulting assembly was allowed to stand undisturbed for 2 weeks, after which time if enzyme crystals had not formed the MPD concentration of the reservoir was incremented by 2%. This process was repeated until crystals appeared. Using this process crystals of the

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1The abbreviations used are: MPD, 2-methyl-2,4-pentandiol; MES, 2-(N-morpholino)ethanesulfonic acid.
enzyme have been seen to first appear at reservoir MPD concentrations between 16% and 24%. The reason for this variation in crystallization conditions is not understood.

Crystals of the enzyme usually grow to a useful size within 2 to 4 weeks after they first appear. Usually several crystals are found in a single well slide. Fig. 1 is a photograph of crystals of the enzyme. It can be seen that the crystals have the appearance of rectangular plates. Their dimensions are typically $0.6 \times 0.4 \times 0.08$ mm. The thin dimension is along the $a$ axis. Crystals as large as 2.5 mm$^2$ in area have been observed, but they are rarely thicker than 0.10 mm. The crystals are stable for at least 1 year in their mother liquor.

Crystals of inorganic pyrophosphatase were mounted in the conventional manner in thin-walled glass capillary tubes that had been pretreated in the same fashion as had been the well slides in which the crystals were grown. These crystals were then examined on Buerger precession cameras in which the crystal to film distance was set at 60 mm. CuK$_a$ x-radiation ($\lambda = 1.5418$ Å) was produced by an Elliot GX-6 rotating anode x-ray generator equipped with pyrolytic graphite monochromators. The electron beam of the x-ray generator was focused to a diameter of 200 pm and operated at a voltage of 50 kv and a current of 50 ma.

The 2-fold symmetry of the crystals' diffraction pattern indicated that they had monoclinic symmetry. The systematic absences of the $0 k 0$ reflections for $k$ odd (assigning the 2-fold axis to be the $b$ axis), together with the fact that the space groups of crystals containing asymmetric molecules such as proteins cannot incorporate centers of symmetry, indicate that the space group of the yeast inorganic pyrophosphatase crystals is P2$_1$. The unit cell dimensions of the crystal are $a = 70.4$, $b = 95.4$, $c = 52.2$ Å and $\beta = 99.8^\circ$ (all $\pm 0.5\%$). The unit cell volume is therefore $3.45 \times 10^5$ Å$^3$.

Figs. 2, A and B are precession photographs of the $0 k 1$ and the $h 0 1$ zones, respectively, of the enzyme crystals. Their diffraction patterns, which can be seen in Fig. 2 to go out beyond spacings corresponding to resolution of 2.8 Å, exhibit significant presence to at least a resolution of 2.5 Å. Successive x-ray photographs of the same pyrophosphatase crystal indicated that the diffraction pattern of such a crystal does not seriously decay for over 30 hours under the above radiation conditions. This is sufficient time to obtain one or two high quality diffraction photographs of the inorganic pyrophosphatase crystals. Therefore, it is clear that the high resolution structural study of yeast inorganic pyrophosphatase is a feasible undertaking.

The density of the enzyme crystals, $\rho$, is 1.15 g per ml as determined from their position of neutral buoyancy in a calibrated bromobenzene-xylene density gradient column (15). The molecular weight, $M$, of the contents of the asymmetric unit of the unit cell of any crystal is given by the formula $M = N \rho V/n$ where $N$ is Avogadro's number, $V$ is the volume of the unit cell, and $n$ is the number of asymmetric units in the unit cell of the crystal. For a crystal of space group P2$_1$, $n = 2$. Therefore, for yeast inorganic pyrophosphatase, $M = 119,500$. The assumption that the asymmetric unit of the crystals contains 1 molecule of an enzyme of molecular weight 68,000 leads to the conclusion that the crystals are 57% protein by weight. This value falls in the center of the range of protein composition of various crystalline proteins that was compiled by Matthews (16). If the asymmetric unit of the unit cell contained 2 molecules of the enzyme, then the composition of the crystals would be 104% protein by weight, a clearly impossible figure. As

![Fig. 1. A photomicrograph of crystals of the yeast enzyme inorganic pyrophosphatase.](image_url)

![Fig. 2. Precession photographs of crystals of yeast inorganic pyrophosphatase. A, the $0 k 1$ zone. The crystal was mounted with its $b$ axis parallel to the spindle axis. B, the $h 0 1$ zone. The crystal was mounted with its $c^*$ axis parallel to the spindle axis. In both photographs the precession angle, $\mu$, was 16.0°.](image_url)
suming a somewhat lower value for the enzyme's molecular weight would, nevertheless, yield an unrealistic proportion of protein in the crystals. It is also not possible that the asymmetric unit of a unit cell of space group P2₁ contain only one protomer of a dimeric protein. This is because it is impossible to place these identical asymmetric subunits in equivalent positions in such a crystal and still maintain the dimeric property of the enzyme molecule. Hence it is concluded that crystals of yeast inorganic pyrophosphatase contain 1 molecule of enzyme in the asymmetric unit of their unit cell.

Crystals of yeast inorganic pyrophosphatase are rather fragile and, therefore, handling them is a somewhat tedious procedure. Cross-linking the crystals for 16 hours in a 0.03 M MES buffer solution that was 30% in MPD and 0.05% in glutaraldehyde (17) appeared to improve their mechanical stability. However, this treatment also caused the crystals' diffraction pattern to vanish beyond a resolution of 4.0 Å. This indicates that the crystal had lost considerable internal order. Lesser exposure to glutaraldehyde decreased this loss of order, but such treatment did not significantly improve the mechanical stability of the crystals.

Avaeva et al. (18) established that yeast inorganic pyrophosphatase is reversibly inactivated by various alcohols. The previously described stock solutions of yeast inorganic pyrophosphatase that are 10% MPD by volume have only 60% of their full enzymatic activity, as was determined using the assay procedure of Cooperman et al. (14). The effect on the enzyme of crystallizing it from MPD solutions in the manner previously described was therefore investigated. Crystals of the enzyme that had been kept for 2 years in their MPD-containing mother liquor were redissolved in water. This completely restored the enzyme's activity (45 Kunitz units per mg (3, 14)). Thus it is clear that crystallization of the enzyme from MPD does not irreversibly damage it. However, the MPD-containing stock solutions of the enzyme do not regain their full enzymatic activity for several hours after they had been diluted 1000-fold with assay medium. Hence it appears that the MPD causes a slowly reversible change in the enzyme.

The x-ray structural investigation of yeast inorganic pyrophosphatase is continuing. The search for isomorphous heavy atom derivatives of the enzyme is currently under way.

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