Acta Cryst. (1994). D**50**, 335–336

Crystallization at low salt concentration and alkaline pH and preliminary crystallographic data for a monoclinic form of yeast enolase.

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(*Received* 23 *August* 1993; *accepted* 21 *January* 1994)

Abstract

Yeast enolase (2-phospho-D-glycerate hydrolyase, E.C. 4.2.1.11) has been crystallized by vapor diffusion from a solution containing 22% PEG 4000, 100 m*M* Tris buffer pH = 9.3, 200 m*M* Li_2SO_4. The crystals are monoclinic with $a = 122.5$, $b = 111.8$, $c = 63.7$ Å, $\beta = 95.6°$, space group $P2_1$ and two dimeric molecules are present in an asymmetric part of the unit cell. Crystals have been successfully transferred to an artificial mother liquor, pH = 7.8, 20 m*M* in Mg^{2+} and 5 m*M* in 2-phospho-D-glycerate. We believe that under these lower salt concentration and more alkaline conditions we should be able to localize the two metal ions that participate in catalysis as well as examine binding of high-affinity inhibitors.

Enolase (2-phospho-D-glycerate hydrolase, E.C. 4.2.1.11) is a glycolytic enzyme that catalyzes the dehydration of 2-phospho-D-glycerate (PGA) to form phosphoenolpyruvate. Because of the small free-energy change for this reaction, *ca* 4 kJ mol^{-1}, the reverse reaction, which is used in gluconeogenesis, is also catalyzed. All eukaryotic enolases and some prokaryotic enolases are dimers consisting of identical subunits (Wold, 1971). In yeast enolase each subunit has 436 amino acids (Chin, Brewer & Wold, 1981; Holland, Holland, Thill & Jackson, 1981), a molecular weight of 46673 Da (Brewer, 1981) and under physiological conditions binds one magnesium cation in the 'conformational' site. Binding of the substrate or inhibitor is necessary to create another metal-binding site often referred to as the 'catalytic' site. A divalent metal in this site is required for catalysis (Faller & Johnson, 1974). At pH values higher than 6.5, a third binding site, called 'inhibitory' because the presence of a metal ion in this site inhibits catalysis, has been found (Elliott & Brewer, 1980). Enolase can be easily deionized and almost any divalent metal ion substituted in the conformational site (Brewer *et al.*, 1983). Nine divalent metal ions confer enzymatic activity, with the natural cofactor being Mg^{2+}, while other ions like Hg^{2+}, Tb^{3+}, Li^{+}, do not.

Recently, the crystal structure of the enolase–Zn^{2+} complex has been determined at 1.9 Å resolution and unambiguously showed three monodentate carboxylic ligands and two water molecules forming an almost perfect trigonal bipyramidal coordination of the metal ion (Lebioda & Stec, 1989). The zinc ion is the second-best activator of enolase and it is believed that its role in the enolase activity closely mimics that of Mg^{2+}. Indeed, the structure of the precatalytic complex enolase–Mg^{2+}–PGA confirmed the trigonal coordination of the conformational metal ion (Lebioda & Stec, 1991). These structures did not reveal the binding site of the second, 'catalytic' or 'inhibitory' metal ion. Also studies of a series of enolase complexes with inhibitors and heavier metal ions carried out by X-ray crystallography did not localize the 'catalytic' site (Lebioda, Stec, Brewer & Tykarska, 1991). Even the structure determined at concentrations of Mg^{2+} as high as 100 m*M*, with 10 m*M* PGA, in acetate buffer pH 6.0, did not show the position of the second metal ion (Zhang, Lebioda & Brewer, 1993). We believe that this is primarily due to the high salt concentration, 2.4 *M* ammonium sulfate, and relatively low pH in the artificial mother liquor of crystals that were used in all crystallographic studies. Indeed, at a 2.0 *M* concentration of ammonium sulfate the K_m for the 'catalytic' metal ion is about 26 m*M* (Lebioda, Stec, Brewer & Tykarska, 1991).

Studies of Cd^{2+} and Mn^{2+} complexes of yeast enolase using $^{113}Cd^{2+}$ NMR and EPR spectroscopy (Spencer, Brewer & Ellis, 1985; Lee & Nowak, 1992*a*) indicated that the ligands at the 'catalytic' site are all O atoms and that the 'catalytic' metal ion appears to be more than 12 Å from the 'conformational' site (Lee & Nowak, 1992*b*). In contrast, studies of the bis Mn^{2+} complex of phosphonoacetohydroxamate in the active site of enolase suggested that the two metal ions are bridged by a single O atom of this very strongly bound inhibitor (Poyner & Reed, 1992). Also, earlier EPR studies of Mn^{2+}–enolase complexes at 77 K had revealed a prominent Mn^{2+}–Mn^{2+} spin-exchange coupling that is normally mediated by a bridging ligand (Chien & Westhead, 1971). Clearly, to understand fully the mechanism of enolase, further studies of the metal-ion binding are needed. With this in mind we have searched for crystallization conditions that would be more conducive towards stronger second and perhaps third metal-ion binding.

Initial experiments were carried out with enolase purified to homogeneity by a modification of the method of Lee & Nowak (1992*b*). Later we used protein obtained by the method of Westhead & McLain (1964). After purification, the enzyme is dialyzed against distilled water for 48 h and concentrated to 8 mg ml^{-1}. The crystals were obtained by vapor diffusion at room temperature in the hanging-drop

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setup. Initially, we obtained twinned plates at pH 8.5. The optimization of the crystallization parameters led us to higher pH where single crystals were obtained. At pH 9.3 the enzyme has about 20% of maximal (pH 7.8) activity (Spencer & Brewer, 1984). Best crystals were formed when a drop containing equal volumes of enolase solution in water and 22% PEG 4000 in 100 m*M* Tris buffer, pH = 9.3, 200 m*M* Li_2SO_4 was equilibrated against 22% PEG 4000. Crystals appeared within 10 d and growth was apparently complete within 3 weeks. The largest crystals were 0.7 × 0.4 × 0.2 mm. Two kinds of morphology for these crystals were observed, one with its twofold axis parallel to the largest face, another with its twofold axis perpendicular to the largest face. The unit-cell dimensions for these forms were the same.

The crystals were characterized using precession photographs and later the R-AXIS II system and *DENZO* software. They are monoclinic with $a = 122.5$, $b = 111.8$, $c = 63.7$ Å, $\beta = 95.6°$. Systematic extinctions for $k = 2n + 1$ observed in the $(0k0)$ reflection set implicated space group $P2_1$. Assuming four dimers per unit cell gives V_m = 2.33 $Å^3\ Da^{-1}$, which is similar to the value 2.79 $Å^3\ Da^{-1}$ found for the tetragonal form of enolase (Lebioda & Brewer, 1984). Since the general position for $P2_1$ has a multiplicity of 2, this suggests two dimers per asymmetric unit. The low-salt form appears to be related to the high-salt form which has $a = b = 124.1$ and $c = 66.9$ Å, $P4_22_12$.

The crystals showed diffraction to 2.1 Å resolution on an R-AXIS II area detector but for technical reasons a data set was collected to only 2.5 Å resolution and the R_{symm} obtained was 6.8%. An attempt was made to check the suitability of the new crystallization conditions to obtain crystals of the enolase complex with the substrate and two metal ions bound per subunit. A crystal was soaked in the capillary (Lebioda & Zhang, 1992) and the native mother liquor (22% PEG 4000, pH = 9.3) was gradually changed to 22% PEG 4000, pH = 7.8, which is the enzyme optimum pH. Subsequently, this artificial mother liquor was made initially 10 m*M* Mg^{2+}, then 5 m*M* PGA, and finally 20 m*M* Mg^{2+}. The solutions were added sequentially with time intervals of 24 h. The crystal survived the soaking process without cracking and diffracted X-rays to at least 2.8 Å resolution on a still photograph. We expect that at this low ionic strength the formation of the quaternary complex enolase–Mg^{2+}–PGA–Mg^{2+} will take place in the crystals and that we will be able to determine the 'catalyic' metal-ion binding site. In addition, we can now study binding of the 'reaction intermediate analogue' phosphonoacetohydroxamate, which binds competitively with a dissociation constant in the nanomolar range at pH values of 9 or higher (Anderson, Weiss & Cleland, 1984).

This work was supported by the National Science Foundation (grant DMB-9018114 to LL).

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