

Acta Crystallographica Section D

**Biological
Crystallography**

ISSN 0907-4449

Editors: **E. N. Baker** and **Z. Dauter**

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Microseed matrix screening to improve crystals of yeast cytosine deaminase

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A crystallization strategy termed 'microseed matrix screening' is described where the optimal conditions for nucleation *versus* extended lattice growth are not compatible. This method is an extension of conventional seeding techniques in which microseeds from the nucleation step are systematically seeded into new conditions where all components of the drop are allowed to vary to screen for subsequent growth of well ordered specimens. The structure of a crystal form of yeast cytosine deaminase produced by streak-seeding using a single condition for both nucleation and growth is compared with the structure of a related crystal form produced by separating nucleation and growth conditions. The resulting structural comparison demonstrates that differential chelation patterns of cations by acidic surface residues of proteins within crystal lattice contacts is a critical parameter of crystal nucleation and growth.

Received 2 October 2003
Accepted 22 December 2003

PDB Reference: yCD crystal form I, 1ra0, r1ra0sf.

1. Introduction

Preparation of macromolecular crystals for X-ray diffraction analysis requires two sequential processes: initial nucleation of the crystal lattice and subsequent three-dimensional growth of a well ordered specimen. In practice, once conditions have been found that produce visible crystals, subsequent trials involve systematic variation of a large number of parameters. However, these follow-up screens are usually performed by additional *de novo* crystallization experiments, so that nucleation and subsequent growth continue to be driven by the equilibration of the crystallization drop against a single reservoir buffer. For some macromolecules, the best buffer compositions for these two processes differ significantly: the conditions leading to nucleation do not promote ordered crystal growth, but unfortunately nucleation does not occur under optimal growth conditions. When a single crystallization buffer recipe cannot efficiently induce nucleation and also promote extended growth of well ordered specimens, the resulting crystals may be unsuitable for diffraction studies. In such cases, each condition must be optimized separately and then combined sequentially (Saridakis & Chayen, 2000).

Previous studies have demonstrated techniques to separate the crystal nucleation and growth phases through the introduction of crystal seeds. These techniques include the transfer of intact crystals or crystal fragments (macroseeding) or the transfer of microscopic crystal nuclei (microseeding) by crushing

existing crystals and streak-seeding (using a fiber to draw across an existing crystal; Stura & Wilson, 1991, 1992). Other studies have concentrated on the introduction of nucleating agents into the growth condition (epitaxial seeding; D'Arcy *et al.*, 2003; McPherson & Shlichta, 1988). The principle in each case involves the introduction of nucleating material into new drops at lower levels of supersaturation where controlled and well ordered crystal growth can occur. The use of seeding techniques is often performed to ensure the isomorphous crystallization of related molecules and can be used in conjunction with other optimization measures such as adjusting the precipitant and protein concentrations or screening additives (Bergfors, 2003). This study extends the use of seeding techniques to include the introduction of nucleants into different crystallization solutions for the improvement of diffraction quality, resolution and/or changing crystallographic space groups of existing crystals.

During the crystallization of yeast cytosine deaminase (yCD), we exploited a method termed 'microseed matrix screening' where nuclei formed using one crystallization condition are systematically transferred to a matrix of different crystallization buffers to screen for a different condition that supports ordered growth. In this secondary screen, individual components of the nucleation buffer (such as the identity and concentration of salts and type of precipitant) are varied systematically. The structure of an initial crystal form of yCD grown directly from nucleation buffer was determined and compared with the structure of

the final crystal form produced from separate sequential nucleation and growth buffers. Coordination of a calcium ion (present only in the growth buffer) at a specific lattice contact point causes a decrease in the length of two unit-cell axes in the final crystal form, corresponding to a 10% reduction in unit-cell volume and a significant improvement in mosaicity and diffraction quality.

2. Methods and results

2.1. Protein expression and initial crystallization conditions

The subcloning, expression and purification of yCD were carried out as described previously (Ireton *et al.*, 2003). Briefly, yCD was overexpressed as a His-tagged protein, purified by batch over Talon metal-affinity resin (Clontech) and further processed to remove the tag by digestion with biotinylated thrombin and passage over a streptavidin column. Selenomethionine-

derivatized protein was expressed according to the method described by Doublé (1997). The proteins were concentrated to 20 mg ml⁻¹, dialyzed into 50 mM NaCl, 20 mM Tris-HCl pH 7.5, 1 mM EDTA, aliquoted and flash-frozen in liquid nitrogen prior to crystallization.

Initial crystallization conditions for yCD were determined by sparse-matrix screening (Jancarik & Kim, 1991) using several commercially available kits (Hampton Research, Emerald Biostructures) as well as in-house combinatorial matrices. The crystallization experiments were conducted using the hanging-drop geometry and vapor-phase equilibration, with 2 µl drops containing 1 µl of 10–15 mg ml⁻¹ protein and 1 µl reservoir solution suspended over 500 µl reservoirs. Crystals were observed in two different conditions at 277 K. The first condition (25% PEG 8000, 0.2 M ammonium sulfate, 0.1 M sodium cacodylate pH 6.5) resulted in multi-lattice clusters that diffracted in house to a d_{\min} value of 2.2 Å. The space group could not be determined

for these crystals owing to the presence of multiple overlapping diffraction patterns. The second condition (28–32% PEG 8000, 0.2 M sodium acetate, 0.1 M sodium cacodylate pH 6.5) produced crystals that grew as twinned clusters on the surface of a heavy precipitate and appeared after three to four weeks, but were difficult to reproduce or to improve through subsequent *de novo* crystallization grids. Sparse-matrix screening with selenomethionine-derivatized yCD resulted in the same crystallization conditions and degree of crystal disorder.

Subsequent streak-seeding experiments were performed from native yCD crystals grown in the second condition described above. The method of seed transfer employed involved using a cat whisker to draw across the face of an existing crystal, then through a pre-equilibrated drop (Stura & Wilson, 1991). Seeds were transferred into 3 µl drops containing 2 µl of 10 mg ml⁻¹ protein and 1 µl 15% PEG 8000, 0.2 M sodium acetate, 0.1 M sodium cacodylate. This drop was pre-equilibrated for 24–48 h at 277 K against reservoirs containing 22–25% PEG 8000, 0.2 M sodium acetate, 0.1 M sodium cacodylate pH 6.5 prior to seeding. This trial produced single crystals that appeared overnight and grew to 0.05 × 0.075 × 0.2 mm in 3–6 d (Fig. 1*a*; crystal form I). Cross streak-seeding these crystals with the selenomethionine-derivatized yCD protein produced crystals that had apparent single lattice diffraction to 2.0 Å and were members of the primitive orthorhombic space group $P2_12_12_1$, with unit-cell parameters $a = 54.3$, $b = 72.5$, $c = 79.4$ Å.

The crystals from this condition diffracted to high resolution at beamline 5.0.2 at the Advanced Light Source Synchrotron facility using a Quantum Q210 area detector (ADSC). The diffraction was strong but quite anisotropic, with spots extending to 1.6 Å along the most ordered axis and to lower resolution (2.5 Å) in other directions (Fig. 1*c*). The spot profiles were somewhat diffuse, with indications of minor alternate lattices or inherent crystal disorder in the diffraction pattern. A selenomethionine-derivatized data set (corresponding to 14 Se atoms per enzyme dimer) was collected. Data were processed and scaled using the *DENZO/SCALEPACK* program package (Otwinowski, 1993; Otwinowski & Minor, 1997). The refined mosaicity for a complete data set was approximately 1.2°. Attempts to solve the structure of yCD using this crystal form *via* multiple or single anomalous dispersion (MAD/SAD) methods (Hendrickson, 1991) were unsuccessful, as selenium sites could not be determined.

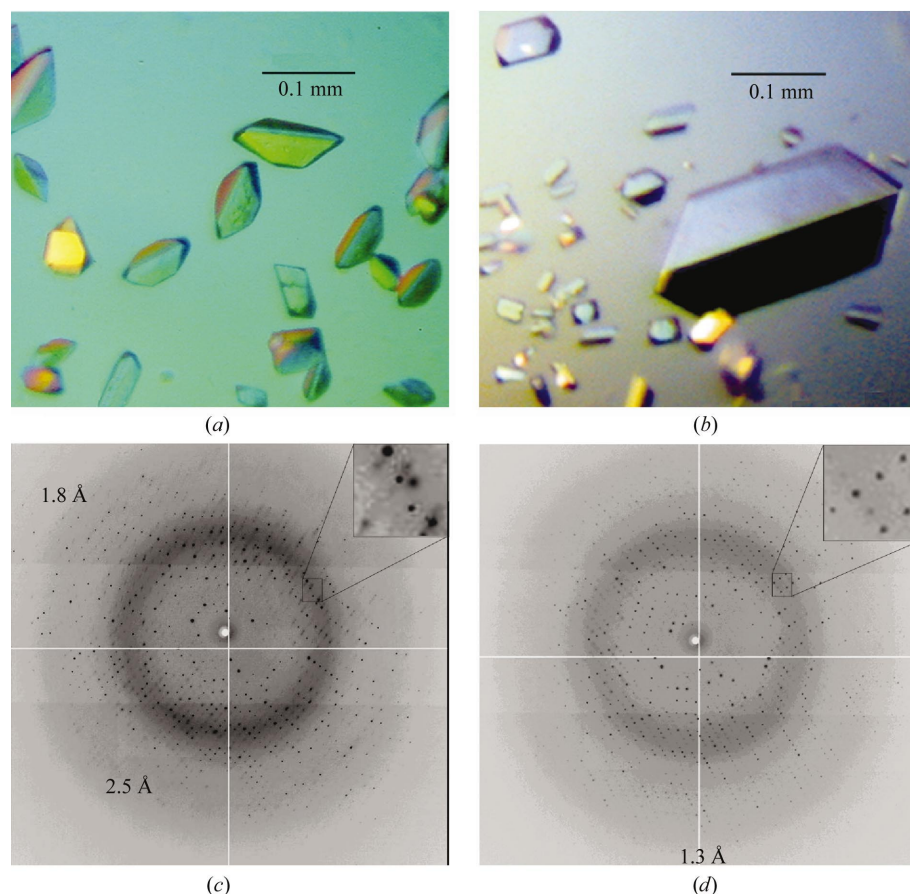


Figure 1

Crystals of yCD. (*a*) Crystal form I grown in presence of sodium acetate. (*b*) Crystal form II grown by microseeding from initial crystal form I into similar crystallization conditions with calcium acetate replacing sodium acetate. The largest crystals grew in the drop distal to the streak line, which was visible. As the amount of seeds transferred was highly variable, some drops were drawn through up to three times, but most just once. (*c*) Diffraction in the presence of sodium acetate. (*d*) Increased resolution and diffraction quality of crystals grown in the presence of calcium acetate.

Table 1
Data-collection and refinement statistics for yCD.

Values in parentheses are for the outermost resolution shell.

Data collection	
Space group	$P2_12_12_1$
Unit-cell parameters (Å)	$a = 54.3, b = 72.5,$ $c = 79.4$
Resolution (Å)	20–2.1 (2.15–2.1)
Completeness (%)	99.9 (99.5)
R_{merge}	0.059 (0.213)
Average $I/\sigma(I)$	34.4 (8.7)
Redundancy	5.8
Refinement	
Resolution (Å)	20–2.1
No. of reflections	18523
Test set (5%)	926
R_{cryst} (%)	18.5
R_{free} (%)	21.1
R.m.s. deviations	
Bonds (Å)	0.005
Angles (Å)	1.10
No. protein atoms	2366
No. water molecules	233
Average B factors (Å ²)	
Protein	29.3
Waters	39.0
Ramachandran distribution (%)	
Most favored	92.0
Allowed	8.0
Disallowed	0.0

2.2. Matrix microseeding

As described above, two closely related crystallization conditions (PEG 8000, 0.1 *M* sodium cacodylate pH 6.5 with either sodium acetate or ammonium sulfate as a salt) produced nucleation events leading to crystal specimens, but the fully formed specimens were poorly ordered. We decided to conduct a broader set of cross-seeding 'optimization' screens for improved crystal growth using the crystals grown under the sodium acetate nucleating conditions as the source for streak-seeding. Parameters systematically varied included the precipitating agent (PEG 400–20 000), buffer composition and pH (5.5–8.5) and counter ions/salts as well as a full complement of commercially available additives and detergents (Hampton).

In one secondary matrix, the salt from the initial conditions (0.2 *M* sodium acetate) was replaced with a series of 0.2 *M* salts with alternative anions and cations. Experiments were conducted under conditions that had resulted in initial crystal nucleation (28–32% PEG 8000, 0.1 *M* sodium cacodylate pH 6.5) as well as conditions optimized for crystal growth (24% PEG 8000, 0.1 *M* sodium cacodylate pH 6.5). Streak-seeding led to crystal growth in a wide variety of salts and in a no-salt control, while no growth was observed in the unseeded drops. Using this strategy, a related crystal form (Fig. 1*b*; crystal form II) was grown by cross-seeding against drops pre-equilibrated with 0.2 *M*

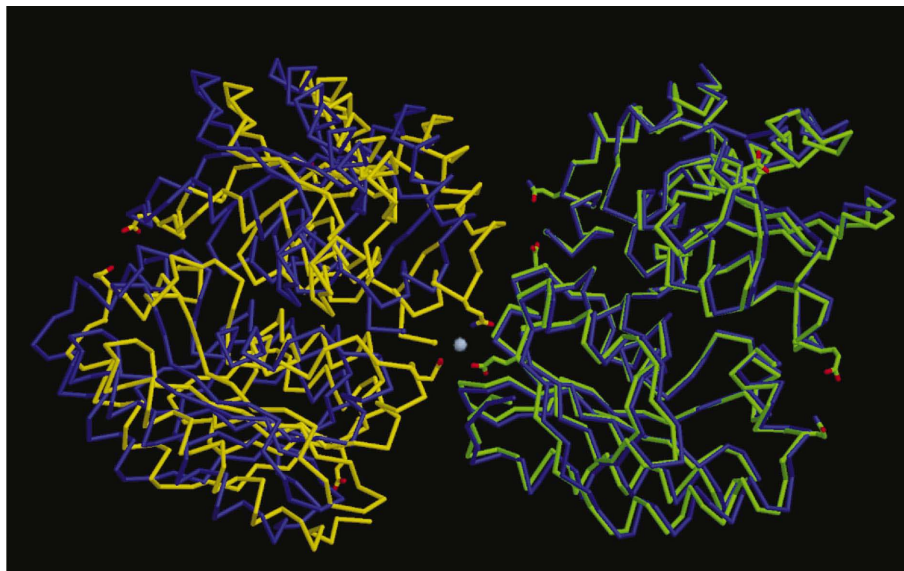


Figure 2
Superposition of dimer packing between adjacent asymmetric for crystal forms I (green and yellow) and II (blue). The presence of bound calcium induces a movement of one enzyme subunit towards its partner in the asymmetric unit. In response, two unit-cell edges display a significant decrease in length (the b and c axes are reduced by approximately 2 and 9%, respectively), leading to a decrease in the unit-cell volume of approximately 10% (27 500 Å³).

calcium acetate in place of sodium acetate in the original buffer. The pH and concentrations of protein and precipitant were unchanged. Direct transfer of the seed crystals to the new condition containing calcium resulted in cracking and disintegration of the specimens. The final condition used for streak-seeding contained 22–25% PEG 8000, 0.1 *M* calcium acetate, 0.1 *M* sodium cacodylate pH 6.5 and produced single crystals that grew to dimensions of 0.15 × 0.2 × 0.4 mm in 3–5 d. Surprisingly, all attempts to grow the new crystal form under these conditions by *de novo* nucleation (without transfer of microseeds) were unsuccessful.

The new crystals are also members of the primitive orthorhombic space group $P2_12_12_1$, with unit-cell parameters $a = 54.4, b = 70.5, c = 71.7$ Å. The substantial reduction in length of the b and c unit-cell parameters corresponds to a reduction of approximately 10% in unit-cell volume. The crystals displayed outstanding diffraction characteristics, including high resolution ($d_{\text{min}} = 1.4$ –1.1 Å), a mosaic spread of less than 0.3° and sharp spot profiles with no indication of minor twinned lattices (Fig. 1*d*). Native yCD crystals grown in the presence of calcium acetate were used to cross-streak the selenomethionine-derivatized protein. The positions of selenium sites were easily determined for this crystal form and the structure was solved using the single anomalous dispersion method at 1.4 Å and with a

mechanism-based inhibitor at 1.1 Å (PDB codes 1ox7 and 1p60, respectively; Ireton *et al.*, 2003).

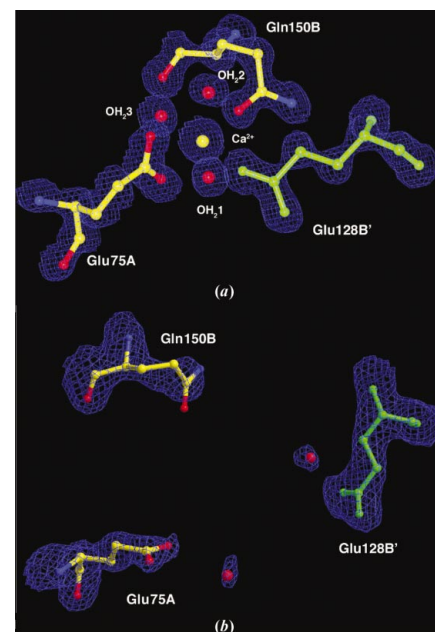


Figure 3
Calcium-mediated lattice contacts in crystal form II and the corresponding region in initial crystal form I. (a) A bound calcium ion is coordinated by surface side chains from three separate enzyme subunits: Glu75A and Gln150B from a physiological dimer and Glu128B' (green) from a subunit of a neighboring asymmetric unit. (b) In the presence of sodium in the initial crystallization condition (form I) no bound cation is visible and the corresponding side chains are further apart.

2.3. Structural comparison

Subsequent to the structure determination of yCD using the final crystal form (Ireton *et al.*, 2003), we retrospectively determined the structure of the enzyme from the original crystal form (*i.e.* grown under a single nucleating condition) using the method of molecular replacement. The structure was determined at 2.1 Å resolution and refined using the program *CNS* with a random 5% of the data excluded for the purpose of cross-validation to a final $R_{\text{work}}/R_{\text{free}}$ of 18.4/21.0 (Brünger, 1993; Brünger *et al.*, 1998). The asymmetric unit contained the biological dimer. In the final refined model, 92% of the residues are in the most favored region of Ramachandran space and the remaining 8% in allowed regions; there are no disallowed backbone angles. Refinement statistics are provided in Table 1.

The structures of the individual subunits are virtually identical, with an r.m.s.d. value of 0.71 Å for all atoms and a side-chain r.m.s.d. of 0.93 Å in the physiological dimer in the asymmetric unit. In the molecular-replacement solution, the first seven N-terminal residues of each yCD monomer are disordered, whereas the structure solved with calcium acetate had observable electron density for both N-termini within the asymmetric unit. An additional three residues corresponding to three residues (Gly-Ser-Ser) remaining on the yCD N-terminus after thrombin cleavage of the N-terminal histidine tag were also observed for one of the two monomers within the asymmetric unit for the calcium acetate crystal form. These three residues are involved in crystal-packing interactions between adjacent asymmetric units. The most significant difference between the structure determined under nucleating conditions (in the presence of sodium) and the optimal structure determined with calcium is the relative positions and packing of individual enzyme subunits in the unit cell (Fig. 2). In the presence of calcium, the individual asymmetric units (composed of enzyme homodimers) are packed tightly together, whereas the same packing is considerably looser in the presence of sodium. The predicted Matthews coefficient increases from 1.95 to 2.25 Å³ Da⁻¹, corresponding to an increase in solvent content from 37 to 45% (Matthews, 1968). When a single enzyme dimer (contents of the asymmetric unit) is superimposed on its counterpart from the alternate crystal structure, the nearest-neighbor asymmetric unit differs in overall position by approximately 5 Å r.m.s.d. This change in packing represents a rigid shift of

the entire asymmetric unit and when propagated through the entire unit cell corresponds to the overall difference in dimensions of the *b* and *c* crystallographic axes.

The tighter packing of enzyme dimers in the presence of calcium is caused by the non-covalent cross-linking of three independent enzyme subunits through the coordination of a single bound calcium ion. The metal is coordinated in an octahedral arrangement by a single surface side chain from each subunit (Glu75A and Gln150B from a physiological dimer and Glu128B' from a neighboring dimer) and three additional water molecules (Fig. 3). In the 1.14 Å data set, a second calcium was also observed, coordinated by Glu75B and Gln150A and four additional water molecules across the dimer interface, although not involved in lattice contacts. The metal-oxygen bond distances in the refined structure range from 2.21 to 2.35 Å. In the absence of calcium, the same side chains are still ordered, but Glu75A, Gln150B and Glu128B' are in different rotameric conformations and are no longer within close enough distance of one another to establish a solvent or salt-mediated bridge.

3. Conclusions

It is somewhat non-intuitive that an outstanding condition for crystal growth would not also be appropriate for nucleation. In vapor-phase crystallization experiments, the concentration of both protein and of precipitating agents increase throughout the experiment until steady state is reached. The tendency of individual molecules to specifically associate into crystal lattice nuclei rather than non-specifically aggregate and precipitate is dictated by at least three factors: the rate at which these concentrations rise during equilibration, the initial and final concentrations after mixing and equilibration, respectively, and the details of the protein solubility and phase behavior in the presence of the crystallization buffer components. In the case of yCD crystallization, the optimal condition for crystal growth, when used for *de novo* crystallization, leads to precipitation. Systematic variance of either the protein concentration or the precipitating-agent concentration failed to enhance nucleation and growth. It appears that crystal growth under these conditions requires slightly elevated PEG 8000 concentrations; unfortunately, the kinetics of equilibration and/or the final steady-state concentrations are inappropriate for nuclei formation.

The use of matrix microseeding in this study resulted in a dramatic increase in crystal diffraction quality and resolution and has also been demonstrated to be an effective means to crystallize proteins in new space groups (Uppsten *et al.*, 2003). In our studies with yCD, numerous crystallization seeding experiments (over 100 trays) were performed in new mother liquors in an attempt to increase crystal-growth quality, facilitated by an abundance of protein material (between 50 and 100 mg of purified yCD were required in order to optimize crystal specimens for data collection). While seeding enabled crystal growth across a range of PEG precipitants (2000 MME–10 000) and salts and counter-ions, the most immediate and dramatic effect on crystal quality was observed by replacing sodium acetate by calcium acetate. We have more recently seen a similar effect on crystal quality in a completely unrelated project in which a divalent magnesium ion is replaced with strontium (BLS, unpublished data). These observations support the idea that cation-mediated crystal packing involving variable chelation patterns by acidic residues found on protein surfaces is a major (and perhaps under-appreciated) variable in protein crystallization projects. The most significant conclusion of this study from a technical standpoint is that care must be taken, even after producing strongly diffracting crystals, to continue to explore broad variations in crystallization conditions. It is also important to exploit the fact that poorly ordered crystals may provide the necessary seeding material for subsequent growth under a different (but usually related) set of conditions, leading to optimized and well diffracting specimens.

We thank Margaret Black and her laboratory for providing the initial clones of yCD and for expert assistance in this project. Funding was provided by the NIH to BLS (GM49857, CA97328) and to GCI (training grant CA09437).

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