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Elimination of Twinning in Crystals of *Sulfolobus solfataricus* Alcohol Dehydrogenase Holo-Enzyme by Growth in Agarose Gels

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Abstract

Crystals of the binary complex of alcohol dehydrogenase from *Sulfolobus solfataricus* with NADH were shown to be twinned and not suitable for automated data collection. Several crystallization trials, performed with the aim of eliminating twinning, are described. Interestingly, crystals grown from agarose gel have been demonstrated to have a unique reciprocal lattice. These crystals are monoclinic, space group *C*2, with cell dimensions $a = 134.47$ (9), $b = 85.26$ (5), $c = 71.76$ (8) Å, $\beta = 97.53$ (4)°, and showed significant diffraction beyond 3.0 Å resolution.

Introduction

Sulfolobus solfataricus is a hyperthermophilic archaeon (Woese, Kandler & Wheelis, 1990; Cowan, 1992) which grows in hot sulfurous mud pools in the mouth of the Solfatara volcanic crater, near Naples. Its optimal growth temperature of 360 K and pH of 3.5 place very stringent requirements on the stability of its proteins which must not merely survive, but operate optimally in these conditions. The very high structural stability of enzymes from thermophilic organisms makes them very attractive for biotechnological applications, and there is considerable interest in understanding their structures.

Sulfolobus solfataricus expresses an NAD^+-dependent alcohol dehydrogenase (*Ss*ADH), formed by two subunits of 37 kDa. The enzyme is active towards a large variety of primary and secondary alcohols, aromatic aldheydes and both linear and cyclic ketones. The protein exhibits extreme thermostability; it is stable and active up to 368 K. Its amino-acid sequence has been determined (Ammendola *et al.*, 1992). *Ss*ADH has been crystallized both in the presence and absence of the coenzyme NAD(H). The apo-enzyme forms large tetragonal bipyramids diffracting beyond 2.89 Å resolution. Structure solution of this crystal form by multiple isomorphous replacement is in progress (Pearl *et al.*, 1993).

Long prismatic crystals of the holo-enzyme (with NADH) have also been grown, but these show a clear physical twinning and give rise to a twinned reciprocal lattice. The twinning renders automated data collection/processing virtually impossible. In order to cope with this serious problem, also recently observed in the ADH holo-enzyme crystals from *Drosophila* (Gordon, Bury, Sawyer, Atrian & Gonzales-Duarte, 1992), we performed a series of crystallization trials, which are reported in this work. Eventually, we were able to grow in agarose gel crystals of *Ss*ADH-$(NADH)_2$ whose diffraction pattern gives clear evidence of the presence of a unique reciprocal lattice.

Experimental

Materials

*Ss*ADH was purified following the protocol of Ammendola *et al.* (1992). Horse liver alcohol dehydrogenase (HLADH) was purchased from Boehringer Mannheim and purified by HiLoad 26/60 Superdex 200 pg FPLC column (Pharmacia). Purity was determined by polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate. All the electrophoresis chemicals were from Bio-Rad. NADH was enzyme grade from Sigma Chemical Company. Tris, trisodium citrate and agarose were Fluka's Biochemika Microselect reagents. All other chemicals were reagent grade. Dialysis buttons for crystallization were supplied by Cambridge Repetition Engineers Ltd.

Crystallization

Screening around the conditions producing twinned crystals. Twinned crystals were grown by vapour diffusion in sitting drops with 2-methyl-2,4-pentanediol (MPD) [50%(*v*/*v*)], Tris/HCl buffer (150 m*M*,

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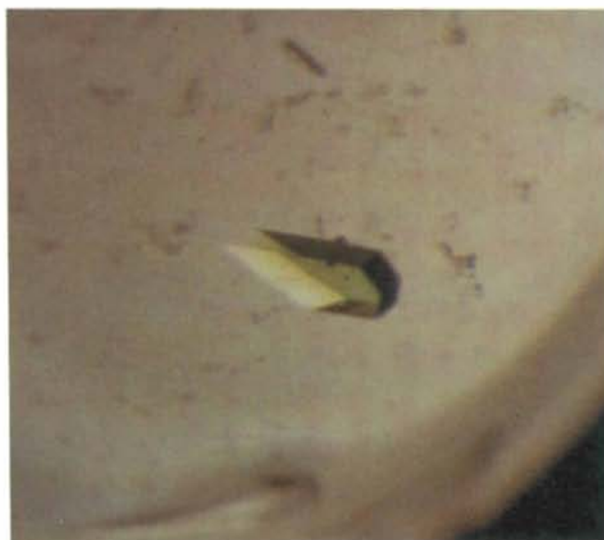
pH 8.4), *Ss*ADH (8 mg ml^{-1}) and NADH (1 m*M*). A screen bracketing these conditions was systematically performed. Precipitant agent concentration [40–55%(*v*/*v*)], buffer concentration (50–150 m*M*), protein concentration (5–15 mg ml^{-1}) and pH (6–9) were varied over the specified ranges at two different temperatures, 277 and 293 K. Finally, addition of dioxane [1–4%(*v*/*v*)] was tried, which poisons the mother liquor. Crystallization trials have been performed by microdialysis also. In all cases prismatic crystals, with the characteristic tailcoat end, were obtained.

Heterogeneous cross seeding. Crystals of the binary complex of HLADH with the reduced form of cofactor were prepared, according to Zeppezauer, Soderberg & Brändén (1967), in the Cambridge microdialysis buttons. One of these crystals was crushed to produce a seed stock solution. The selected seed crystals of about 0.1 mm, after extensive washing, were introduced into pre-equilibrated sitting drops of a *Ss*ADH(NADH)$_2$ solution, at 277 K. The protein concentration was 7 mg ml^{-1} in Tris/HCl buffer (10 m*M*, pH 8.0), the precipitant agent was MPD [36 or 38%(*v*/*v*)] in Tris/HCl buffer (50 m*M*, pH 8.4) plus NADH (1 m*M*). The seeding produced crystals (up to 1.2 × 0.5 × 0.3 mm) within a week.

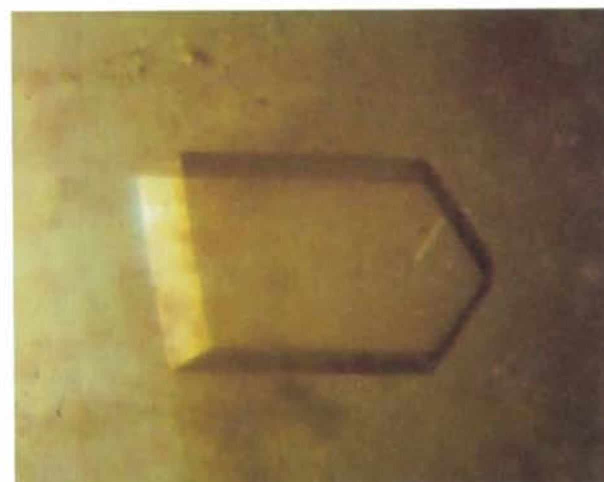
Use of agarose gels. The agarose mother solution and the crystallizing agent solutions were prepared according to Robert, Provost & Lefaucheux (1992). The best crystals were obtained by mixing 5 μl of a protein solution containing *Ss*ADH (7 mg ml^{-1}) in Tris/HCl buffer (10 m*M*, pH 8.0) with 5 μl of a crystallizing agent solution of MPD [40%(*v*/*v*)], agarose [0.05%(*w*/*v*)], Tris/HCl buffer (50 m*M*, pH 8.4) and NADH (1 m*M*). The gelled hanging drop-

(a)



(c)

(b)



(d)

Fig. 1. Crystals of *Ss*ADH(NADH)$_2$: (*a*) twinned crystals with a tailcoat end; (*b*) poorly diffracting crystals; (*c*) twinned crystals obtained by cross-seeding technique; (*d*) un-twinned crystals obtained in agarose gel.

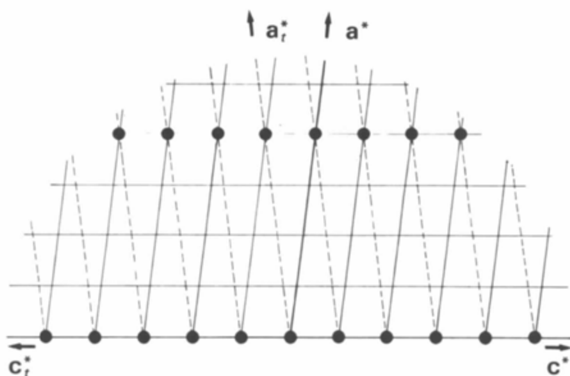


Fig. 2. Reciprocal lattice scheme of the $C2$ monoclinic twinned crystals of *Ss*ADH(NADH)$_2$ seen along the unique axis. The two lattices are related by a 180° rotation around the a axis. Dots (●) indicate the positions of reflections $8n,0,l$ of one lattice and $8n,0,-l-n$ of the twin lattice (broken line), respectively.

lets were equilibrated with 1 ml of a reservoir solution of MPD [40%(v/v)] and Tris/HCl buffer (50 mM, pH 8.4) at room temperature. Prismatic crystals appeared and grew to maximum dimensions of 1.0 × 0.4 × 0.2 mm within one month.

New crystal form of SsADH(NADH)$_2$. Prismatic crystals were grown at 277 and 293 K by microbatch and free interface diffusion methods. A solution containing *Ss*ADH (24 mg ml^{-1}) in Tris/HCl buffer (130 mM, pH 8.0) was mixed with (or layered over) a solution containing NADH (2 mM), polyethylene glycol (PEG) 4000 [16%(w/v)], propan-2-ol or propan-1-ol [16%(v/v)] in trisodium citrate (100 mM, pH 4.8–5.6). The largest crystals (0.4 × 0.3 × 0.2 mm) were obtained with trisodium citrate pH 4.8 with an effective pH value in the crystallization samples of approximately 5.9.

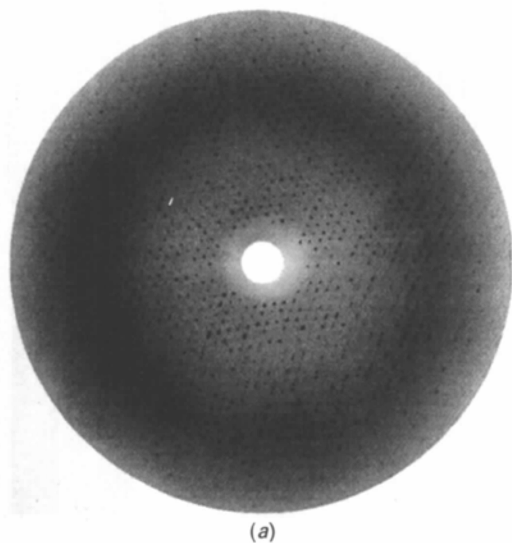
X-ray data

The unit-cell parameters $a = 131.5$, $b = 85.8$, $c = 70.8$ Å, $\beta = 97°$, of the twinned $C2$ holo-enzyme were previously determined from precession photographs (Pearl *et al.*, 1993). Using these data, the orientation of a crystal grown in agarose gel was determined, and 20 × 1° oscillation images collected on a MAR image plate, mounted on a Siemens rotating-anode source, were successfully processed using the *MOSFLM* package (Leslie, 1992).

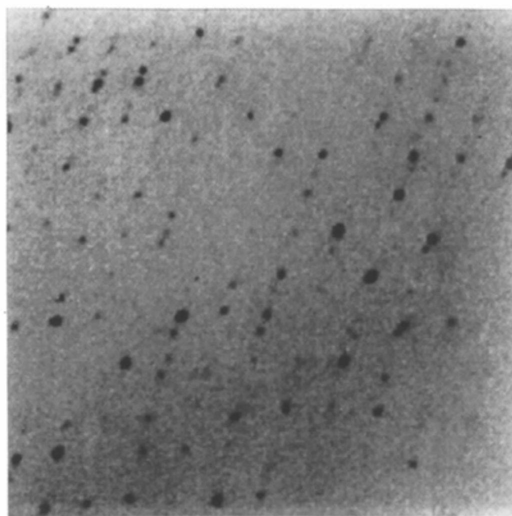
For all diffracting crystals, the oscillation images and/or the $h0l$ precession photographs have been carefully examined to recognize the twinning.

Results

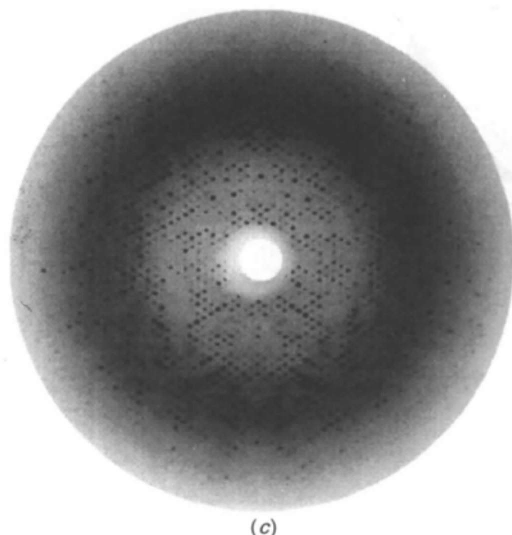
A detailed analysis shows the diffraction pattern of the twinned crystals (Fig. 1a) to result from a mono-



(a)



(b)



(c)

Fig. 3. 1° Oscillation photographs of *Ss*ADH(NADH)$_2$ crystals: (a) crystals grown by cross-seeding; (b) close-up image of (a) showing the occurrence of twinning; (c) crystals grown in agarose gel.

clinic $C2$ lattice superimposed upon an identical lattice related to the first by a 180° rotation around the a axis. The resulting pattern displays mm symmetry in $h0l$ lattice section with splitting of the spots parallel to c^*, as shown in Fig. 2. The combination of the unit-cell parameters and the direction of the twinning axis produces an almost exact superposition of the reflection $(8n,k,l)$ of one lattice with the $(8n,-k,-l-n)$ reflection of the twin lattice.

In order to obtain an un-twinned form of the complex with NADH, we have explored a large variety of other crystallization conditions and obtained prismatic crystals in conditions similar to those for the apo-enzyme (Fig. 1*b*). Disappointingly, these crystals diffract only weakly to a little beyond 4 Å resolution, and are not suitable for data collection.

Earlier observations of the twinned $C2$ holo-enzyme form showed that the degree of twinning varied with temperature, with a generally lower degree of twinning in crystals grown at 277 K, although still too much for data processing. This suggested that the twinning may be due to secondary nucleation giving rise to epitaxial growth of a secondary crystal. The effect of lower temperature may then be to lower the degree of convection in the sample and limit secondary nucleation events. This possibility was explored in a series of experiments in which holo-enzyme crystals were grown in similar conditions to those producing the twinned crystals, but enhancing the control of the nucleation step.

On this basis, the poisoning of the crystallization medium with dioxane, which in some cases eliminated the twinning (Sigler, Jeffery, Matthews & Blow, 1969; Watson, Wendell & Scopes, 1971), the variation of the protein and/or precipitant agent concentration, and the variation of the dilution of precipitant in the droplet were attempted unsucessfully to eliminate the twinning.

To decouple nucleation and crystal growth steps, the seeding technique has been used, with seeds of $HLADH(NADH)_2$. The inspection of the diffraction pattern of crystals (Fig. 1*c*), obtained by this technique, also revealed twinning in this case (Figs. 3*a*,*b*).

Finally, crystallization in agarose gels was attempted. This method usually provides for the slow diffusion of the solute or solvent into the gel, and inhibits secondary nucleation, lowering convective flows and sedimentation effects. Crystals of SsADH$(NADH)_2$, grown in agarose gels, lacked the clear physical twinning often observed in holo-enzyme crystals grown by conventional means, suggesting they are un-twinned (Fig. 1*d*). This was confirmed by X-ray diffraction of a large prismatic crystal (0.8 × 0.4 × 0.2 mm), which gave a unique reciprocal lattice (Fig. 3*c*). Rotation images from these crystals could be easily processed giving data of good quality. The cell parameters determined from post-refinement of the rotation images were a = 134.47 (9), b = 85.26 (5), c = 71.76 (8) Å, β = 97.53 (4)°. This gives a V_M of 2.76 $Å^3$ Da^{-1} with one dimer in the asymmetric unit. The crystal showed significant diffraction beyond 3.0 Å resolution.

Although growth in agarose gels has eliminated the twinning phenomenon which had previously prevented data collection of this holo-enzyme form, several of the crystals grown in this way completely failed to show any diffraction pattern whatsoever, and were presumably disordered. Apparently, only crystals freshly extracted from the gelled droplet and soaked in a stabilizing solution prior to excision from the gel and mounting, showed diffraction. For this reason, further work is in progress to analyse the effect of various factors on the diffraction power of crystals grown in gel medium.

Our experimental data suggest that growth in gels can be useful from crystals in which multiple nucleation and twinning are problems. However, the handling of gel-grown crystals may generate new problems of its own so that additional data are required to draw some meaningful conclusions.

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