SHORT COMMUNICATIONS

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A designed mutant of the enzyme glutathione reductase shortens the crystallization time by a factor of forty.

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Abstract

The packing of glutathione reductase from *Escherichia coli* in crystal form *T* showed a place where two molecules are at a distance of only 6 Å between the closest atoms, *i.e.* where a contact is almost made. In order to form this contact with hydrogen bonds, two amino-acid residues were exchanged. This mutation had no effect on molecular packing or the resolution limit of the X-ray diffraction, but facilitated crystal nucleation dramatically and possibly increased the crystal growth rate and shortened the crystallization time.

Introduction

X-ray structure analyses depend on suitable crystals. For proteins these crystals should diffract X-rays to 2 Å resolution or beyond. In numerous cases, however, only crystals with resolutions around 3 Å can be obtained. Such crystals suffice for establishing the polypeptide-chain fold and the general locations of the side chains, but mostly fail to answer the chemical questions of interest. Knowing the approximate geometry of the packing contacts at 3 Å resolution, these contacts can be modified by mutations in order to improve the crystal quality. Such an improvement has been reported for ferritin, where an adverse species difference was remedied (Lawson *et al.*, 1991).

With crystal form *T* of the enzyme glutathione reductase from *Escherichia coli* we encountered a situation suitable for contact engineering. This crystal form diffracts X-rays to 2.8 Å resolution and shows a packing scheme where one contact is missed by only a couple of ångströms. In an attempt to improve the crystal quality, we have bridged this gap by introducing larger side chains at two positions.

Materials and methods

Wild-type and mutant glutathione reductases from *E. coli* were produced as recombinant enzymes expressed from the *gor* gene in the vector pKK223-3 in a strain of *E. coli*, NS3, which lacks the chromosomal glutathione reductase gene (Deonarain, Berry, Scrutton & Perham, 1989). The enzymes were purified as described by Berry, Scrutton & Perham (1989). Invariably, the enzyme was passed through a 2′,5′-ADP-Sepharose affinity column shortly before setting up the crystallization trials.

The mutant was produced by the phosphorothioate method (Taylor, Ott & Eckstein, 1985) using the mutagenic oligonucleotide 5′-ACGTTGATCCACAGCCGTACCTA-CTATATCGA-3′, and the gene sequence was checked by dideoxy sequencing (Biggin, Gibson & Hong, 1983). The mutant gene was then subcloned into the expression vector pKK223-3 as previously described (Scrutton, Berry & Perham, 1987).

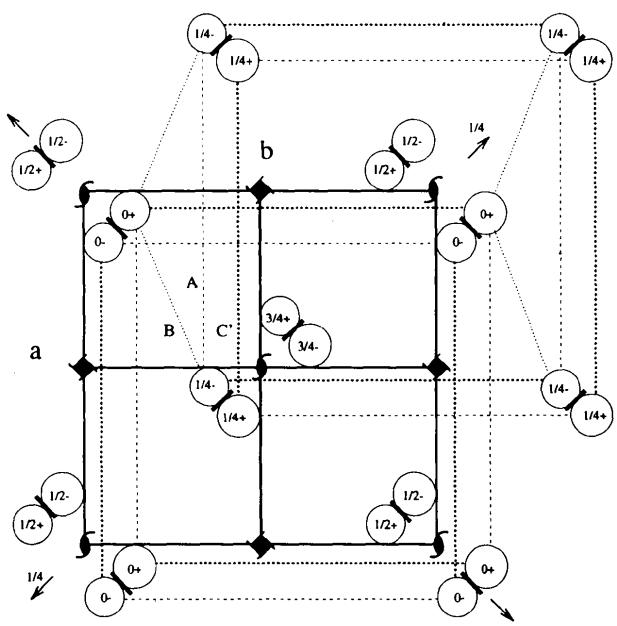

Fig. 1. Packing scheme of crystal form *T* of glutathione reductase from *E. coli*. The crystals belong to space group $P4_32_12$ with one subunit per asymmetric unit. The subunits are sketched as circles containing the height specification along the *c* axis. The heights and all symbols are given as in *International Tables for Crystallography* (1983, Vol. A). The dimer interfaces are indicated by bars between the circles. Contacts *A* (thin dashed line) and *B* (thin dotted line) suffice to form the three-dimensional lattice. Contact *C'* (thick dotted line) is absent in the wild-type, but should be formed in the mutant enzyme crystals.

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Glutathione reductase from *E. coli* is a homodimer with a subunit M_r of 49 560. Each subunit contains 450 amino-acid residues and one FAD molecule. All crystallizations were carried out in hanging drops at 293 K. The crystallization procedures and conditions for wild-type and mutant enzymes were as follows: an enzyme solution of about 20 mg ml^{-1} with a volume of 0.1 ml was dialyzed overnight (membrane Servapor, cut-off M_r 10 000–15 000) against 50–100 ml 20 m*M* potassium phosphate buffer at pH 5.4 containing 5% PEG-10000 (Fluka) and 0.02% NaN_3. The enzyme solution was then centrifuged at 14 000 *g* for 10 min and applied as 5 µl drops onto siliconized glass plates. The glass plates were inverted and mounted above 0.6 ml of a reservoir solution, which contained 20 m*M* potassium phosphate at pH 5.4, 20% PEG-10000 (Fluka) and 0.02% NaN_3. Crystal sizes were measured visually using a microscope with a graticule. Two crystallization trials of the mutant enzyme were documented photographically.

For determining solubilities, a protein solution (50 µl, 30 mg ml^{-1}) was dialyzed against 20 m*M* potassium phosphate buffer at pH 5.4 (50 ml). The dialyzed solution was titrated with 20% PEG-10000 (Fluka) in this buffer. The protein solution was visually inspected 15 min after each titration step. Each titration experiment was performed three times.

The crystals of wild-type and mutant enzymes were of form *T*, belonging to space group $P4_32_12$ with axes $a = b = 62.0$ (2), $c = 337$ (1) Å and one subunit per asymmetric unit. They were isomorphous and grew with identical habits, pointed quadratic bipyramids. The resolution limits of the X-ray diffraction patterns of the wild-type and mutant enzymes in crystal form *T* were determined photographically using Ni-filtered Cu $K\alpha$ radiation from a rotating-anode generator (model RU200B, Rigaku).

The structure of the wild-type enzyme in crystal form *T* has been solved by molecular replacement (unpublished results) using the wild-type enzyme structure previously established in crystal form *P*, first at 3.0 Å resolution (Ermler & Schulz, 1991) and then at 2.0 Å (unpublished results). The structure in crystal form *T* was further refined using *X-PLOR* (Brünger, Kuriyan & Karplus, 1987) to an *R* factor of 32.9% in the resolution range 9.0–3.1 Å. The molecular packing is illustrated in Fig. 1. The dimer interface and contacts *A* and *B* determine the three-dimensional lattice. Contact *C'* is almost made in the wild-type and was modelled to form in the engineering mutant.

Results

Fig. 2(*a*) shows the region of the wild-type enzyme in crystal form *T* where the contact *C'* is almost formed. The closest distances Ala86-CB···Asp365-O and Ala90-CB···Asp366-CB are both 6 Å. By introducing the mutations Ala86→His and Ala90→Tyr, these gaps should be bridged and should give rise to a contact with two hydrogen bonds (Fig. 2*b*). It was hoped that this additional contact would solidify the three-dimensional lattice illustrated in Fig. 1, thereby improving the crystal order.

The Ala86→His/Ala90→Tyr mutant was purified in an identical manner to the wild-type enzyme, and kinetic analysis showed that the two mutations had not affected the enzymatic properties. Initial crystallization attempts with this mutant indicated that the crystallization times were appreciably shortened compared with the wild type. This behaviour was subsequently quantified by monitoring crystallization time and crystal size for about 30 hanging drops each of the wild-type and the mutant enzymes. These drops were set up in batches of four to eight drops on different days in order to reduce unforeseeable handling

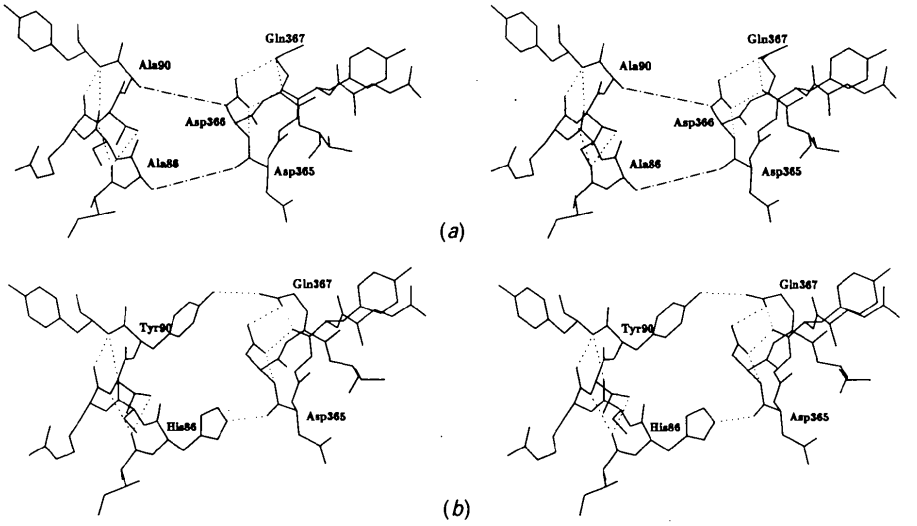
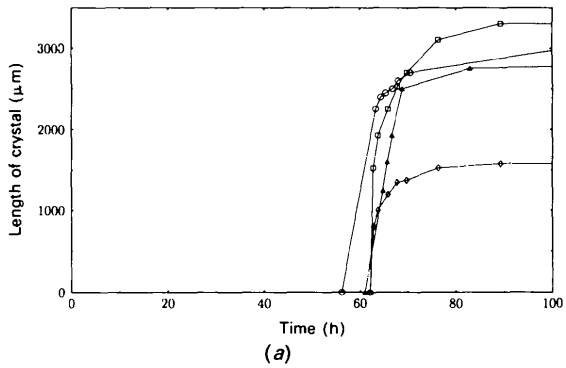
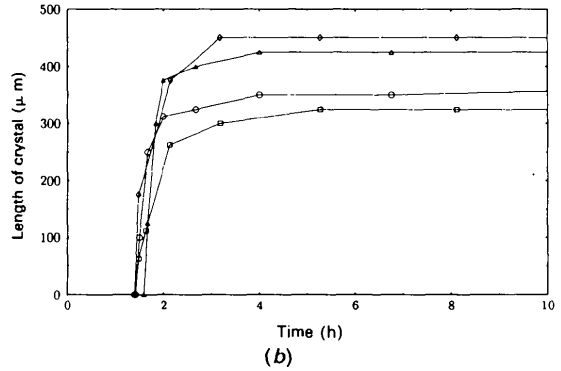


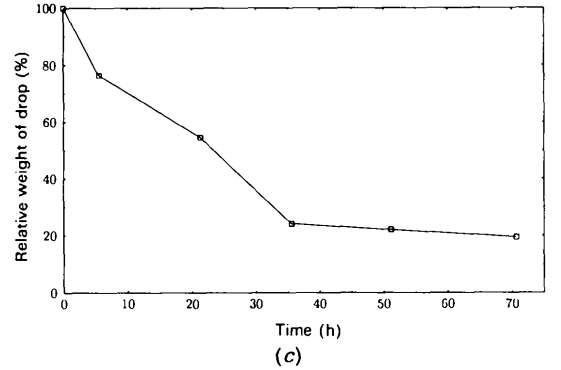
Fig. 2. Atomic structure around crystal contact *C'*. (*a*) The structure of the wild-type enzyme in crystal form *T*. This structure has been solved by molecular replacement at a resolution of 3.1 Å and subsequently refined using *X-PLOR*. The distances Ala86-CB···Asp365-O and Ala90-CB···Asp366-CB (dash-dot lines) were both found to be around 6 Å. (*b*) Model of Ala86→His/Ala90→Tyr mutant built into the wild-type structure such that crystal contact *C'* is formed by hydrogen bonds His86-NE2···Asp365-O and Tyr90-OH···Gln367-OE2 (dashed lines) with lengths around 3.0 Å. In the model all atoms of the wild-type enzyme except the side chain of Gln367 have been kept in their original places.



(*a*)



(*b*)



(*c*)

Fig. 3. Crystallization series of wild-type and designed mutant enzyme using the hanging-drop method. (*a*) Growth curves in the four drops of the series that yielded crystals of the wild-type enzyme. The longest dimension between the two apices of the quadratic bipyramid is plotted as a function of time. These four drops contained one single crystal each. In the drop designated o crystal growth started overnight. Therefore, the starting point of the growth curve was taken as the time-average between the last check showing no crystal and the first check showing a crystal. (*b*) The same as (*a*) but with the designed mutant enzyme forming contact *C'*. Note the time scale of the plot. (*c*) Gas-diffusion progress in a crystallization drop of 5 μl. The weight of the drop (starting around 5 mg) is plotted as a function of time. Because of the large weighing errors the data for the four monitored drops were put on a relative scale and averaged. As the drop weight decreases to 20–25% of the initial value, the PEG-10000 concentration increases from the initial 5% into the range around 20%, which corresponds to the PEG-10000 concentration of the reservoir.

influences. All successful attempts at growing crystals in this series of experiments are given in Fig. 3. Crystals only grew in four drops of the wild-type and in four drops of the mutant enzyme.

For the wild-type enzyme the start of crystallization occurred reproducibly around 60 h after being set up, in agreement with previous qualitative experience with the wild-type enzyme and with several mutants containing changes at places other than at the crystal contacts. As shown in Fig. 3(*a*), the crystals grew to 70% of their maximum size within 2–5 h of the start of crystallization.

In contrast, the designed mutant crystallized reproducibly about 1.5 h after being set up, and the crystals grew to 70% of their maximum sizes in less than 1 h. In both cases the behaviour is surprisingly uniform. In this series of experiments the wild-type produced discrete crystals which grew to very large sizes in each drop, whereas the mutant formed numerous small crystals. This difference is not significant, however, because in preceding crystallizations the mutant enzyme had yielded large single crystals with longest dimensions around 2000 μm.

The Ala86→His/Ala90→Tyr mutant had been designed to form the additional *C'* crystal contact in the hope of achieving a better crystalline order, in turn resulting in X-ray diffraction to higher resolution. Therefore, we recorded on photographic film the diffraction patterns of three wild-type and two mutant crystals of comparable sizes under similar X-ray beam conditions. For all crystals, the pattern extended to 2.8 (2) Å showing no significant difference between wild-type and mutant enzyme. This series of measurements confirmed our prior impression of these crystals; that the mutation had failed to improve their crystalline order.

In order to check their stability, wild-type and mutant crystals were first transferred to reservoir buffer with 20% PEG-10000, where they are stable, and then introduced into the same buffer with lower PEG-10000 concentrations. These stability tests revealed a sharp limit: wild-type and mutant crystals were stable for at least one month at PEG-10000 concentrations down to 5.3% and dissolved within 1 d at 5.0%. The additional contact of the mutant made no difference in crystal stability.

This observation correlates well with the solubilities of the wild-type and mutant proteins: in three titration experiments for each, wild-type and mutant enzyme, the protein solutions remained clear for PEG-10000 concentrations up to 5.2%. At concentrations above 5.5%, invariably the wild-type enzyme precipitated and the mutant enzyme crystallized within 15 min. Accordingly, the solubilities are identical and they meet the crystal stability limit.

To correlate the time of crystal formation with the increase in the PEG-10000 concentration above the starting value of 5% in the drop, we monitored the sizes of four drops over time by weighing them (Fig. 3*c*). On average, the PEG-10000 concentration in the drop equilibrated after 35 h in the 20–25% range. After 1.5 h, when the mutant starts to crystallize, the concentration was about 5.5%.

Discussion

The observed data demonstrate that the mutation Ala86→His/Ala90→Tyr changed neither crystalline order nor solubility and crystal stability, but showed a profound

effect on the starting point of crystallization, which occurred 40 times faster. In addition, the mutation may have increased the crystal growth rate. This point was difficult to establish, however, because the growing surfaces of the numerous smaller mutant crystals were larger than those of the single wild-type crystals, which may well explain the observed rate difference of a factor of about three in this series of experiments (Figs. 3*a*,*b*).

The gas-diffusion progress in the crystallization drops (Fig. 3*c*) indicated that the mutant enzyme succeeds in nuclei formation shortly after the PEG-10000 concentration crosses the solubility and crystal stability limits of about 5.3%, requiring almost no supersaturation. In contrast, the wild-type enzyme requires a high and enduring supersaturation for nucleation. Wild-type nuclei form 60 h after the drop crosses the solubility and crystal stability limit, which is 25 h after the drop has equilibrated at a high level of supersaturation (Fig. 3*c*). Most surprising is the uniform timing in all eight successful crystallization trials of the series (Figs. 3*a*,*b*).

In conclusion, we find that the addition of contact *C'* accelerated the crystal nucleation process, shortening the crystallization time by a factor of 40. The engineered contact may have increased the crystal growth rate. It did not affect protein solubility, crystal stability and crystalline order. The experiments demonstrate that contact engineering can have a strong influence on crystallization, nurturing the hope that it can be used for improving the crystalline order in the future.

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Crystallization and preliminary investigation of xylose isomerase from *Bacillus coagulans*.

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Abstract

Xylose isomerase from *Bacillus coagulans* has been crystallized in two different crystal forms. One crystal form is in space group $P2_12_12$, cell dimensions $a = 462$, $b = 165$, $c = 82$ Å. The other is in space group $I422$, cell dimensions $a = b = 113$, $c = 153$ Å.

Introduction

Xylose isomerase, XI (E.C. 5.3.1.5) catalyzes *in vivo* the isomerization of D-xylose to D-xylolose (Hochster & Watson, 1953). This activity is known to involve two divalent ions (Mg^{2+}, Mn^{2+}, Co^{2+}) per molecule (Kasumi, Hayashi & Tsumura, 1982).

This paper describes the crystallization and preliminary investigation of xylose isomerase from *Bacillus coagulans* (BXI).

The crystallographically determined structure of XI from a number of other species is known: XI, *Actinoplanes missouriensis*, 2.8 Å resolution (Rey *et al.*, 1988); XI, *Streptomyces olivochromogenes*, 3.0 Å resolution (Farber, Glasfeld, Tiraby, Ringe & Petsko, 1989); XI, *Arthrobacter*, 2.5 Å resolution (Henrick, Collyer, Blow, 1989); XI, *Streptomyces rubiginosis*, 1.9 Å resolution (Carrell *et al.*, 1989; Collyer, Henrick & Blow, 1990); XI, *Streptomyces albus*, 1.65 Å resolution (Dauter, Terry, Witzel & Wilson, 1990); XI, *Streptomyces rubiginosus*, 1.6 Å resolution (Whitlow *et al.*, 1991).

The sequence of BXI differs remarkably from the other known species, as does the optimum activity and stability. While the activity of *Streptomyces* species increases with increasing pH without reaching optimum activity, BXI has an activity optimum at pH 8.2. A similar phenomenon is seen for activity as a function of temperature between 313 and 263 K. No temperature optimum is found for *Streptomyces* species while BXI has its optimum at 358 K (personal communication, Novo Nordisk A/S). An alignment of the sequence of BXI to the sequences of the other species using the program *FASTP* (Lipman & Pearson, 1985) of the package *PIR* (*Protein Identification Resource*; National Biomedical Research Foundation, 1991) only gives about 25% sequence identity.

BXI contains 442 residues per monomer giving a calculated molecular weight of 50 180 Da.