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CRYSTALLOGRAPHIC STUDIES ON *E. coli* Trp APOREPRESSOR**

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Two crystal forms of Trp aporepressor, an inactive, unliganded form of Trp repressor have been obtained which are suitable for high resolution X-ray diffraction analysis. Trp aporepressor crystallizes in two forms: orthorhombic, P 2_12_12 and tetragonal P 4_1 (or P 4_3) which diffract to 1.8 Å and 2.4 Å, respectively. The orthorhombic crystals contain one monomer in the asymmetric unit, therefore the twofold axis relates two subunits of the dimer as in the case of the previously described Trp repressor (R. Schevitz *et al.*, 1985, *Nature*, **317**, 782 - 786) **and Trp pseudorepressor** (C. Lawson & P. B. Sigler, 1988, *Nature*, **333**, 869 - 871). **The tetragonal crystals have two dimers in the asymmetric unit and are nearly isomorphous with the tetragonal crystals of Trp repressor and Trp pseudorepressor grown under similar conditions but in the presence of an activator or inhibitor, respectively.**

Structures of four proteins (catabolite activator protein, operator binding domain of λ repressor, *cro* repressor and Trp repressor) which regulate gene expression have been solved to high resolution by X-ray crystallography [1 - 4]. The low resolution structure of the DNA binding domain of *lac* repressor has been interpreted using n.m.r. [5]. In all these proteins, the helix-turn-helix structural motif has been found. This domain has been shown to bind specifically target DNA sequences [6 - 9]. Direct evidence for specific DNA binding by this domain has been obtained recently from high resolution crystal structure of the Trp repressor-operator complex [10] and similar DNA containing complexes of phage λ cI repressor [11] and phage 434 cI repressor [12]. Numerous studies suggest that mutations in the

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helix - turn - helix region of various proteins affect DNA binding [7 - 9]. Sequence homology and structural similarity of the DNA binding motif have also been noted [6, 4].

Activity of some DNA regulatory proteins is modulated by effector molecules. It has been shown for Trp repressor and catabolite activator protein that sequence homology with gene regulatory proteins in the helix - turn - helix motif is not sufficient for specific DNA binding [1, 4]. Trp repressor and catabolite activator protein can bind tightly and specifically DNA only in the presence of a specific activator molecule, L-Trp or cAMP, respectively.

Structural and biochemical analysis of the Trp repressor system provide detailed information on contribution of the activator molecule. In the Trp repressor, the activator molecule is bound to the helix - turn - helix DNA binding motif and the high resolution structure of Trp repressor - operator complex shows direct involvement of the ligand in specific DNA binding [10]. A modification of L-tryptopan has been reported which alters operator binding drastically [13]. On the basis of high resolution crystal structures of the Trp repressor, aporepressor, pseudorepressor and the complex repressor - operator the molecular model of ligand binding and protein activation has been proposed [10, 14]. The question remains open what changes in the aporepressor structure are induced specifically by the activator molecule and what changes are due to binding to specific and nonspecific DNA. Determination of the Trp aporepressor structure was a very important step in understanding of the molecular switch that controls gene expression. However, the problem is much more complex and will require a more detailed structural analysis. In this report we describe a method for growing two different crystal forms of Trp aporepressor. One crystal structure has been solved recently by Zhang *et al.* [4]. The second crystal structure is nearly isomorphous with crystals of active, unliganded repressor and inactive, liganded pseudorepressor.

MATERIALS AND METHODS

Trp aporepressor was obtained from an overproducing strain of *E. coli* CY 15007 containing multicopy plasmid pRLK18 (kindly supplied by Prof. C. Yanofsky) and purified according to the procedure described earlier [15]. In the final step we replaced separation on hydroxyapatite by chromatography on Amicon Red matrix from Amicon. L-Tryptophan D-tryptophan and indole-3-propionic acid were purchased from Aldrich; norleucine was obtained from Pierce. Crystals were grown by vapor diffusion technique using hanging drop method, and on depression slides in boxes [15, 16, 18]. A microseeding technique was applied to control the nucleation

process [16 - 18]. The best crystals were obtained from 1.95 M ammonium sulfate and 0.5 M sodium, potassium phosphate or potassium citrate at pH 7.0 - 7.5 at room temperature. Both orthorhombic and tetragonal crystals grow very slowly. After one to three months of undisturbed growth they reach the final size of $0.7 \times 0.4 \times 0.15$ mm for orthorhombic semicircles and $1.5 \times 0.1 \times 0.1$ mm for tetragonal rods. Purity of the crystals was checked by h.p.l.c. chromatography on Brownlee RP C-8 Aquapore 300 column under denaturing conditions, in the presence of 0.1% trifluoroacetic acid. Well formed crystals were washed quickly with 100 µl of protein free stabilizer solution and dissolved in 100 µl of 0.1% trifluoroacetic acid. A sample was heated for 2 min at 90°C, cooled on ice and injected to preequilibrated column. Protein was eluted with a linear gradient of acetonitryl. The h.p.l.c. profile was compared to separately run standards of Trp aporepressor and L-tryptophan.

The Trp aporepressor molar absorption coefficient at 280 nm is $2.95 \times 10^4\ M^{-1}\ cm^{-1}$ and was established by amino-acid analysis of pure Trp aporepressor solution of known absorption in the presence of norleucine as internal standard.

Sensitivity of aporepressor crystals to the presence of L-tryptophan and indole propionate was demonstrated by soaking crystals in protein free stabilizer solution containing ammonium sulfate in concentration preventing dissolution of crystals, and 2 mM ligand. As a control, solutions containing 2 mM D-tryptophan as a ligand were used.

RESULTS AND DISCUSSION

Crystals of Trp aporepressor can be grown reproducibly from concentrated salt solutions at neutral pH. We reported earlier growth of Trp aporepressor microcrystals [15]. After applying refined crystallization conditions and introducing concentrated phosphate or citrate anions we have grown single, large, well ordered crystals. Two kinds of crystals have been obtained under the same conditions. Orthorhombic crystals grow as almost perfect semicircles (Fig. 1A) in space group P $2_12_12_1$ [a = 44.5(±0.2)Å, b = =57.3(±0.3) Å, c=34.0(±0.1) Å]. Assuming the density of 2.3 $Å^3$/D for a typical protein crystal [16] we estimate that one half of the dimer occupies the asymmetric unit with four protomers in the unit cell. Since Trp aporepressor exists in solution as a dimer [15], this suggests that two monomers of the dimer are related by the crystallographic dyad similarly as it was shown for the Trp repressor and pseudorepressor [4, 14, 17, 19]. Orthorhombic crystals diffract X-rays to at least 1.8 A resolution (Fig. 2) and exhibit appreciable resistance to radiation damage. To check whether the Trp aporepressor crystals were L-tryptophan sensitive, we soaked crystals

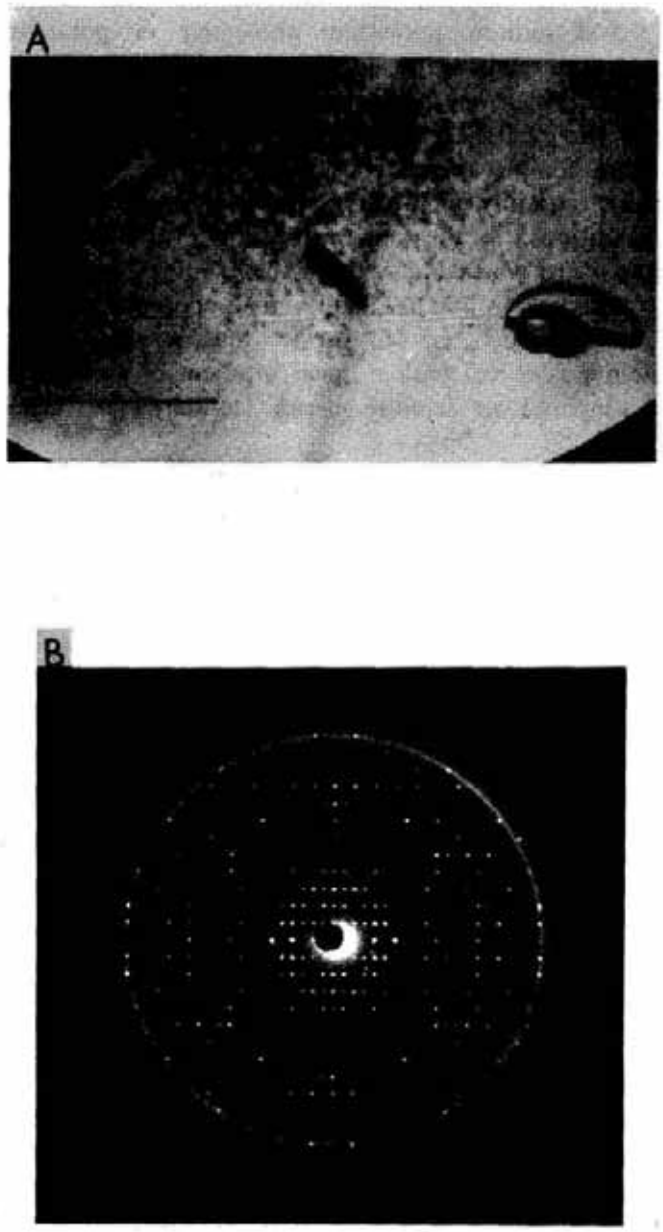

Fig. 1. Orthorhombic crystals and X-ray diffraction pattern of Trp aporepressor. CuKα graphite monochromated X-rays were generated on Elliot GX6 rotating anode at 40 mA/40 kV: A, orthorhombic crystals of Trp aporepressor grown as described in Materials and Methods, bar is 1 mm; B, 16 degree ($d_{min} \geq 2.8$ Å) precission photograph of the (*h0l*) reciprocal lattice plane of the orthorhombic crystal shown in A, exposure time 36 h

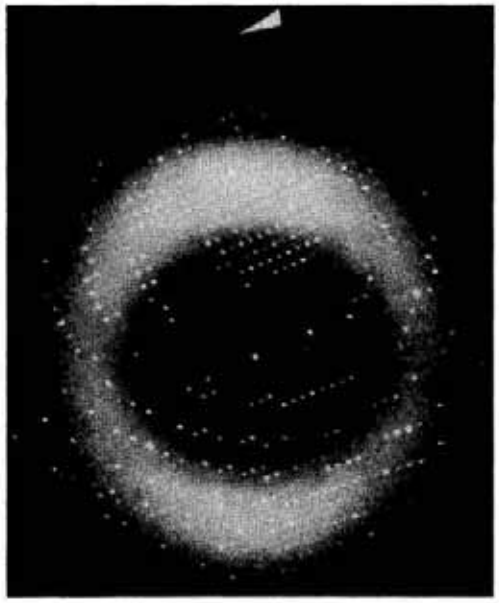

Fig. 2. X-ray diffraction pattern of the Trp aporepressor orthorhombic crystal. X-rays were generated as described in Fig. 1. 3.5 degrees oscillation photograph (3.5 h/degree) with b axis along the spindle, offset by 0.5 degree from the axis perpendicular to the X-ray beam. Data were collected on a film in cylindrical cassette, r = 75 mm. Arrow points to d_{min} = 1.9 Å reflection

in stabilizing solutions containing L-tryptophan or indole propionate. Crystals exposed to L-tryptophan shatter within minutes and the protein precipitates inside the crystal. We observed this same effect in the presence of indole propionate. In a control solution containing D-tryptophan, crystals were stable for a month. Our observations imply that the protein in the crystal undergoes a conformational change of sufficient magnitude to destroy crystal packing and to lower the protein solubility. This result also suggests that orthorhombic crystals contain Trp aporepressor in a conformation different from that of active Trp repressor (e.g. the complex of Trp aporepressor/L-tryptophan) and inactive Trp pseudorepressor. This can not be simply explained by a contribution of L-tryptophan to crystal packing because it has been shown that bound L-Trp is buried inside the protein and does not contribute directly to crystal packing [4]. We have shown previously that, under the conditions used for the aporepressor, the protein in the presence of L-tryptophan or tryptophan analogs crystallizes in a matter of hours exclusively in the tetragonal form [17, 18]. All these observations have been confirmed by analysis of crystal structures of aporepressor [14] and pseudorepressor [19] however, contribution of crystal packing to the structure has not been examined in detail.

Surprisingly, Trp aporepressor in the absence of any ligand can also crystallize in tetragonal crystal form (cf. Fig. 3a) under the same conditions as the orthorhombic form. In fact, very often both crystal forms can be seen in the same drop. The space group is $P\bar{4}$ ($P4_3$), [$a = b = 81.7(\pm 0.2)$ A, $c = 75.2(\pm 0.3)$ A]. Assuming a typical protein density 2.3 A^3/D [16] there

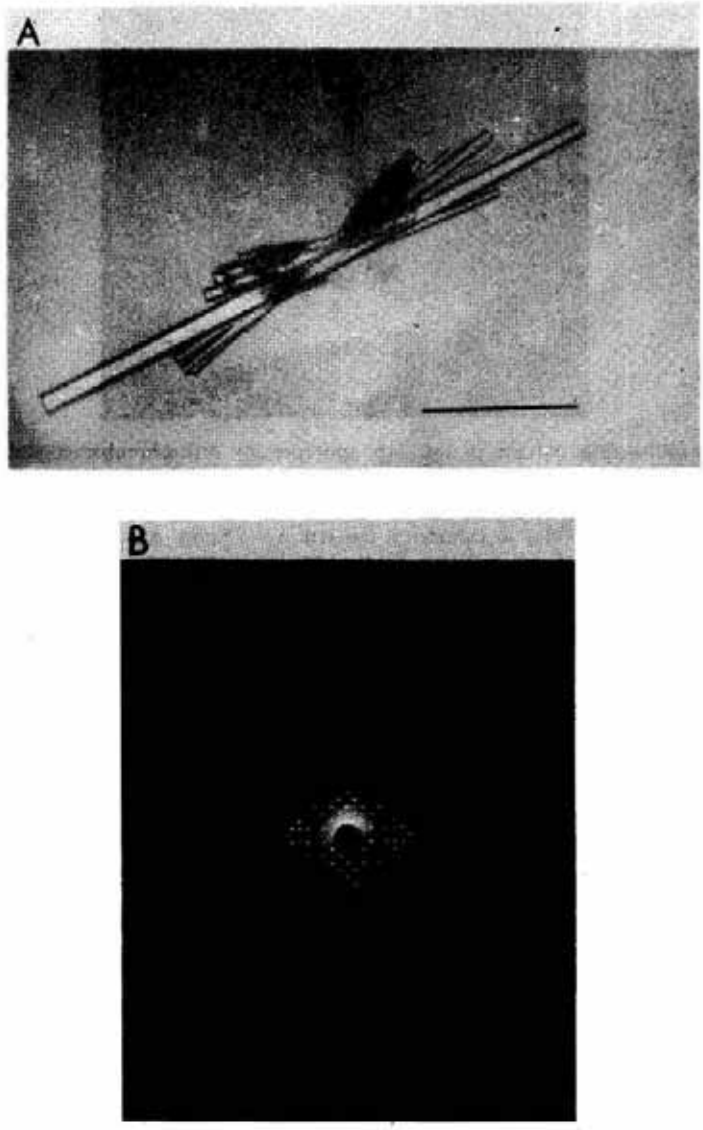

Fig. 3. Tetragonal crystals and X-ray diffraction pattern of Trp aporepressor: **A**, tetragonal crystals of Trp aporepressor grown as in Fig. 1. bar is 1 mm; B. 16 degree ($d_{min} \geq 2.8$ A) precession photograph of (*h0l*) reciprocal lattice plane of the tetragonal crystals shown in **A**, exposure time 72 h. X-rays were generated as described in Fig. 1

are two dimers per assymetric unit and eight dimers in the unit cell. These crystals are virtually isomorphous with tetragonal crystals of Trp repressor and pseudorepressor. The space group of the tetragonal form of Trp repressor and pseudorepressor is P 4_1 (P 4_3) rather than P 4_122 (P 4_322) (based on analysis of upper-level photographs) as we reported earlier [17]. We checked the purity of the tetragonal crystals of aporepressor on reverse phase chromatography for possible contaminations. Like in the case of orthorhombic crystals we could not detect L-tryptophan or any u.v. absorbing tryptophan analog in the crystal. Tetragonal crystals of aporepressor are also sensitive to L-tryptophan and indole propionate. Since these aporepressor crystals do not contain bound ligand, we can conclude that: (i) in this space group crystal packing forces stabilize the protein conformation (lattice specific) which aporepressor can assume with or without bound ligand; (ii) high concentrations of phosphate or citrate stabilize the conformation; (iii) aporepressor can undergo structural transition without active participation of ligand. It has been shown that Trp repressor protein exhibits high degree of flexibility of DNA binding domains [20]. When overproduced in *E. coli* it can regulate additional genes [21]. These results indicate that, in *E. coli*, there are DNA sequences which can be recognized specifically by the repressor although with lower affinity. It is clear from the crystal structure of the repressor/operator complex [10] and orthorhombic aporepressor [14] that Ala77/Val substitution leading to superrepressor [8] contributes directly to organization of the tryptophan binding pocket, and it might stabilize the "active" Trp repressor conformation without presence of the ligand.

The Trp aporepressor structural transition from inactive to active state in solution presumably is not energetically favorable, unless it is helped by bound ligand or altered amino-acid side chain. Otherwise Trp aporepressor would exhibit high affinity towards its operator. Since repressor binding to operator alters protein and DNA structure [10], lattice contacts in the crystal can also apparently contribute sufficient energy to stabilize a lattice--specific protein conformation. However, it is not clear why protein chooses to form under the same conditions two distinctly different crystals, containing the protein in two not identical conformations. In tetragonal crystals it is a state almost isomorphous with that of Trp repressor and pseudorepressor.

Our data provide evidence for at least two stable states of Trp aporepressor observed in orthorhombic and tetragonal crystals. This is consistent with the discovered flexibility of helix - turn - helix motif [20]. The activator molecule might function by shifting equilibrium towards a state (or states) which, upon binding the operator sequence, will adopt the best fit with DNA duplex. In aporepressor such a transition requires too much energy. The difference in growth rate of protein crystals in the absence and presence

of indole ligands (at least two orders of magnitude) could indicate the existence of such mechanism.

Small differences in structure factors between all three tetragonal crystal forms suggest that, although these structures are very similar, they are not identical and might differ locally. These small changes are apparently sufficient to explain the difference in affinity to operator DNA by three to four orders of magnitude, as it was proposed for Trp repressor and pseudorepressor (T. Wagner *et al.*, in preparation).

We now know that the function of L-tryptophan is complex ([13], A. Joachimiak *et al.* in preparation). L-Tryptophan stabilizes orientation of the DNA binding structural motif and molds amino-acid side chains on the DNA binding surface of repressor [4, 10]. However, to better understand the process of Trp aporepressor activation, we need to analyse high resolution structures of various Trp aporepressor crystal forms and compare them with models of Trp repressor, superrepressor and the repressor/operator complex [10]. Detailed structural analysis of all these protein conformations should reveal the molecular details of the conformational switch which controls activation of aporepressor. However, isomorphism between active and inactive state of the protein suggests that its specificity is achieved by local, small but critical changes.

The structure determination of the orthorhombic crystal form has been completed recently [14] and of the tetragonal crystal form is in progress.

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