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Crystallization and Preliminary X-ray Analysis of Recombinant Bovine Cellular Retinoic Acid-Binding Protein

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Abstract

Crystals of bovine cellular retinoic acid-binding protein (CRABPI) have been grown from protein expressed in *E. coli*. Two different crystal forms were obtained. Crystals containing protein with the ligand all-*trans* retinoic acid belong to space group $P4_1$ or $P4_3$, $a = b = 41.36$, $c = 202.71$ Å and diffract to 2.5 Å. Crystals of CRABP with the synthetic retinoid analogue Am80 diffract to 1.9 Å with space group $P2_1$ and cell dimensions $a = 37.03$, $b = 105.93$, $c = 40.31$ Å, $\beta = 110.28°$. Considerations in the crystallization of proteins with light-sensitive ligands are discussed.

Abbreviations

CRABP, cellular retinoic acid protein; CRBP, cellular retinol-binding protein I, CRBPII, cellular retinoic acid II; RA, retinoic acid; RBP, serum retinol-binding protein; PEG, polyethylene glycol.

Introduction

Retinoid-dependent biological functions include embryonic development, spermatogenesis, vision, cell proliferation and differentiation. Their roles in the modulation of mitogenic tumor promoters and cell transformation have focused attention on them as potential anti-carcinogens. Among the retinoids, there are at least two classes of protein which interact with retinoic acid. These are the retinoic acid receptors (Ragsdale, Petkovich, Gates, Chambon & Brockes, 1989; Zelent, Krust, Petkovich, Kastner & Chambon, 1989) and the 15000 Da cytostolic proteins, CRABPI and II. The function of CRABP is unclear. The possible functions which have been suggested are: (1) the metabolism of RA from retinol; (2) the transport of RA through the cytoplasm to its nuclear receptors (Takase, Ong & Chytil, 1986; Dolle, Rubert, Leroy, Morriss-Kay & Chambon, 1990); and (3) the storage of excess RA (Maden, Ong, Summerbell & Chytil, 1988).

CRABP belongs to a protein family with a ten-stranded β-barrel topology enclosing small hydrophobic ligands (for a review, see Banaszak *et al.*, 1994). Cellular retinol binding protein (CRBP) and *P*2 myelin, also members of this family, have been crystallized previously and the structures solved in our laboratory (Cowan, Newcomer & Jones, 1993; Jones, Bergfors, Sedzik & Unge, 1988). Serum retinol-binding protein (RBP) (Newcomer, Jones *et al.*, 1984) belongs to another family of hydrophobic ligand-binding proteins with a similar motif (eight-stranded β-barrel) but with no sequence homology to the CRABP family. CRBPI and CRBPII preferentially bind retinol (Levin, Locke, Yang, Li & Gordon, 1988; Li *et al.*, 1991) but will also bind retinal and even retinoic acid (Ninomiya, Suganuma, Paik, Muto & Fujiki, 1988) whereas CRABP binds only retinoic acid. *P*2 myelin binds retinol, retinoic acid, oleic and palmitic acids *in vitro* (Uyemura, Yoshimura, Suzuki & Kitamura, 1984), although its *in vivo* ligand seems to be a mixture of the latter two (Cowan *et al.*, 1993).

Based on the *P*2 myelin structure, Jones *et al.* (1988) suggested that in the cellular retinoid-binding proteins, the functional group of the retinoids would be situated roughly in the center of the ligand cavity. The ligand specificity of these proteins would then be mediated by the amino-acid side chains which project into the internal cavity of the β-barrel (Jones *et al.*, 1988). In *P*2 myelin, two arginines and a tyrosine residue interact with the carboxylate of the fatty acid. These arginines are conserved in most of the fatty acid members of the family but are replaced by glutamine residues in CRBP and CRBPII. Subsequent structure work has confirmed that one of these glutamines interacts with the retinol alcohol functional group in both proteins (Cowan *et al.*, 1993; Winter, Bratt & Banaszak, 1993). Thus in CRBP and CRBPII, the specificity is primarily determined by a glutamine side chain pointing into the barrel whereas in CRABP (as in *P*2 myelin), there are two arginines and a tyrosine in this position.

Mutagenesis of these residues in CRBP (Stump, Lloyd & Chytil, 1991), CRBPII (Cheng *et al.*, 1991) and CRABP (Zhang, Liu, Jones, Gierasch & Sambrook, 1992) alters the ligand-binding properties. For example, mutating the two arginines in

CRABP to glutamines decreases the protein's affinity for retinoic acid but makes it more thermostable. We have purified and crystallized CRABPI for structure determination to understand further the ligand specificity of these proteins and the structural basis of retinoid function.

Materials and methods

Expression of apo-CRABP in E. coli

The plasmid construction was generously provided by Dr Jianhua Zhang, UTSW at Dallas, USA. The *E. coli* strain JM101 was transformed with the plasmids pGP1-2 (Tabor & Richardson, 1985) and pT7 1-3/CRABP (Zhang *et al.*, 1992), then plated onto LB agar plates containing 100 μg ml^{-1} ampicillin and 40 μg ml^{-1} kanamycin. A 10 ml preculture was grown overnight from the freshly streaked plates at 303 K in 2 × YT medium with appropriate antibiotics. This pre-culture was used to innoculate 2 × 400 ml cultures in SR (super-rich) medium. These were grown to OD_{555} 4–5 at 303 K, then stored at 277 K overnight. The next day a 100 l fermentation with SR medium and antibiotics was innoculated with 0.5 l from these cultures, then grown to an OD_{555} of 3.0 at 303 K (4–5 h). Induction was effected by raising the temperature to 310 K after which the fermentation was continued for another 12 h. 3.25 kg cells (final OD_{555} = 55) were harvested by centrifugation, frozen in liquid nitrogen as a paste with 10% glycerol, and could be stored at 183 K for up to 2 years. A single 100 l fermentation provided all the protein for this study.

CRABP purification

50 g aliquots of cells were thawed at 277 K and lysed in 10 ml of lysis buffer. The lysis buffer contained 20 m*M* Tris–HCl pH 8.0, 50 m*M* β-mercaptoethanol, 1 mg ml^{-1} lysozyme, 4 m*M* phenylmethylsulfonyl fluoride, and 500 units Benzonase (Merck, 25 units μl^{-1}). The solution was stirred until the viscosity decreased significantly (1–2 h) after which it was centrifuged to remove particulate matter. The supernatant was diluted to 500 ml with 20 m*M* Tris–HCl, pH 8.0, 3 m*M* β-mercaptoethanol and submitted to a pre-equilibrated ion-exchange column (Pharmacia's Fast Flow Q Sepharose, 5 × 15 cm). The column was eluted at 100 ml h^{-1} with a 0–0.25 *M* NaCl gradient, with CRABP appearing at 0.1 *M* NaCl. Protein from 4–5 such ion-exchange runs was pooled, concentrated on an Amicon YM3 filter, and applied to a G50 column equilibrated in 50 m*M* Tris–HCl, pH 8.0, 1 m*M* β-mercaptoethanol (Pharmacia, 2.6 × 100 cm, flow rate 12 cm h^{-1}). Fractions from a single G50 run were further purified in ten runs on a Mono Q HR 10/10 (Pharmacia) equilibrated in 20 m*M* Tris–HCl, pH 8.0 with a 0–0.25 m*M* NaCl gradient, then pooled and concentrated.

Purity was assayed by silver staining a 20% homogenous polyacrylamide–SDS gel (Phast System, Pharmacia). The protein appeared as a single band even when heavily overloaded. Silver-stained isoelectric focusing gels, however, revealed one predominant band at pH 4.7 and at least two other faint bands.

Preparation of retinoic acid–CRABP

All manipulations of retinoic acid-bound protein were performed in the dark. 35 mg of purified apo-CRABP was incubated by gentle shaking with a 1–10 molar excess of all-*trans* retinoic acid (Sigma) freshly dissolved in ethanol, either overnight at 277 K or 1.5 h at room temperature. Excess ligand and ethanol were removed by re-applying the protein to the G50 column described above. When the protein was re-concentrated, absorbances at OD_{280} and OD_{350} were measured. Ligand-saturated protein gave a value of 2.07 for the ratio $OD_{350/280}$. This is higher than the value of 1.8 reported previously (Chytil & Ong, 1984), but was obtained consistently for all our batches, once the protein was highly concentated.

Preparation of Am80–CRABP

Because of the inherent difficulties in working with the light-sensitive retinoic acid, crystallization attempts were made with three synthetic light- and oxidation-stable retinoid analogues, TTNN, Am80 and TTAB, generously provided by Dr Siegfried Keidel (Keidel, Rupp & Szardenings, 1992). Only the Am80 analogue produced crystals, however.

Am80 is a synthetic retinobenzoic acid which was developed, along with other retinoid analogues (the so-called super-retinoids), in attempts to find retinoid compounds less cytotoxic than retinoic acid for use in clinical applications. Our interest in it stemmed from its property of light stability. Am80 is at least equally (Tamura *et al.*, 1990) if not more (Jetten *et al.*, 1987; Takagi *et al.*, 1988), biologically active than retinoic acid although it has a lower binding constant for CRABP than the native ligand. The K_i for retinoic acid binding to CRABP is 0.21 μ*M* with reports given of 3.6–9 μ*M* for Am80 (Jetten *et al.*, 1987; Takagi *et al.*, 1988).

Am80 was dissolved in ethanol and Am80–CRABP prepared in a similar manner to retinoic acid–CRABP but without the precautions to minimize its exposure to visible light. Binding of the Am80 ligand increased the absorbance of the protein at OD_{280} by a factor of 1.7 but gave no peak at OD_{350}, this peak being unique for retinoic acid.

Crystallization

Crystallization was carried out by the sitting-drop vapor-diffusion method (McPherson, 1982) in microbridges (Harlos, 1992). All experiments were performed at 277 K.

The crystallization drop of Am80–CRABP contained 3 μl of the protein at 12 mg ml^{-1} in 50 m*M* Tris–HCl pH 8.0, 1 m*M* β-mercaptoethanol, 0.1% β-octyl glucoside; 3 μl of 20% polyethylene glycol (PEG) 4000 (gas chromatography quality, Merck) in 0.2 *M* ammonium sulfate; and 1 μl 10% ethylene glycol. The drop was equilibrated against a 1 ml reservoir of 20% PEG 4000 in 0.2 *M* ammonium sulfate. Crystals appeared within 1 week and often reached sizes of 0.2 × 0.5 × 1.20 mm within 2 months.

Crystals of retinoic acid–CRABP were obtained as follows: 3 μl of the protein (10 mg ml^{-1}) in 50 m*M* Tris–HCl, pH 8.0, 1–2 m*M* β-mercaptoethanol, and 0.1–0.2% β-octyl glucoside were mixed with 3 μl of the reservoir. The reservoir contained 1 ml of 30–37.5% PEG 8000 (Aldrich), 0.1 *M* hepes pH 7.8, 0.2 *M* unbuffered sodium acetate and 5% isopropanol. Crystals grew as long rods (typical size 0.8 × 0.2 × 0.2 mm) in 1 week. Crystallization dishes were wrapped in aluminium foil to prevent exposure to light. Retinoic acid–CRABP crystals fluoresced under UV light, indicating that the fluorescent ligand retinoic acid was indeed present in the crystals.

Crystals have also been grown of the apo-CRABP but have not been characterized yet.

Space-group determination

The Am80–CRABP crystals diffracted to 1.9 Å, belonged to space group $P2_1$ with cell dimensions $a = 37.03$, $b = 105.93$, $c = 40.31$ Å and $\beta = 110.28°$. Data were collected on a Nicolet Xentronics area detector and data evaluated with the program *XDS* (Kabsch, 1988).

Retinoic acid–CRABP crystals belonged to space group $P4_1$ (or its enantiomorph $P4_3$) with cell dimensions $a = b = 41.36$, $c = 202.71$ Å. These diffracted to 2.5 Å. Data were collected on a Rigaku R-AXIS IIC area detector (Sato *et al.*, 1992) and evaluated with Z. Otwinowski's program *DENZO* (Department of Molecular Biophysics and Biochemistry, Yale University, New Haven CT, USA, unpublished). The space group was also confirmed by precession camera pictures.

Results

Several protocols for the purification of CRABP expressed in *E. coli* have been reported (Fiorella & Napoli, 1991; Zhang *et al.*, 1993; Fogh, Voorhees & Åström, 1993; Liu, 1993). In our case we originally modeled the purification strategy on the protocol for recombinant CRBPII (Li, Locke, Yang, Ong & Gordon, 1987) but without success because ammonium sulfate precipitation of the protein resulted in irreversible aggregation. Fiorella & Napoli (1991) reported that pretreatment of the CRABP lysate with DNase was essential for CRABP recovery, and the addition of Benzonase (Merck) also greatly improved our yields. Adding retinoic acid to the cell lysate interfered with protein binding to the anion-exchange material and, therefore, the protein was purified in its apo-form.

To produce ligand forms of the protein, aliquots of the purified apo-protein were incubated overnight with either a 10 molar excess of the ligand or a 1:1 molar ratio. Crystallization drops were then set up. These produced only showers of thin crystals or extremely thin plates with both ligands. It was only after excess ligand and ethanol were removed by passing the protein over a G50 column that large single high diffracting quality crystals could be obtained. Removal of excess ligand by gel filtration was also critical for the successful crystallization of the human growth hormone–receptor complex (Ultsch, de Vos & Kossiakoff, 1991).

Once the retinoic acid–CRABP was obtained from the second gel-filtration column and concentrated, it could be stored at 277 K in lightproof Eppendorf tubes for up to 7 months without affecting its ability to produce good crystals. The $OD_{350/280}$ ratio was measured at regular intervals during this time to monitor possible degradation of the ligand. Over several months this value decreased from 2.07 to 1.93. However, if left exposed to light, this value decreased from 2.07 to 1.88 in less than an hour. After 16 h exposure to light, the ratio fell to 1.09 (see Fig. 1). Protein with an $OD_{350/280} < 1.8$ could crystallize but the crystals were of poorer morphological quality and diffracted to only 6 Å. Crystals grown in the dark, then exposed to the light for even a few hours suffered similar deterioration in their diffraction patterns although they remained morphologically intact. Thus, it was essential to the success of this crystallization project that extreme care be taken to minimize the exposure of both the protein and the crystals to light. These precautions did not seem necessary for the crystals of holo-CRBP (Newcomer *et al.*, 1981) or holo-RBP (Newcomer, Liljas, Sundelin, Rask & Peterson, 1984), perhaps indicating that retinol is not as light sensitive as retinoic acid, or at least not when complexed to the protein. However, Winter *et al.* (1993) report that in the case of retinol–CRBP II, crystallization was performed in the dark although no statement was made about attempts to crystallize the holo-form of the protein in the light.

Although crystals grew best at 277 K, once mounted they were not temperature sensitive. Crystals could grow at room temperature or 288 K, but were not good enough to mount. Because of condensation problems with hanging drops, sitting drops were used at 277 K. Plastic microbridges were silanized with a water-soluble silane (Aquasil, Pierce) to prevent the crystals from sticking to the plastic, a problem in the case of the Am80–CRABP crystals. However this resulted in thin hair-like crystals. When the microbridges were not silanized, hair-like crystals would sometimes appear but large single crystals were in the majority. Streak seeding (Stura & Wilson, 1992) the Am80–CRABP was not required for crystal growth but provided the largest sized crystals. Oddly, these did not grow along the streak line as one might have expected. Streak seeding or microseeding of the retinoic acid–CRABP had no effect on the growth of these crystals.

The protein was purified in the presence of a reducing agent and β-mercaptoethanol was present in the crystallizations. However, crystals grew just as well without it. CRABP has three free cysteines but they are relatively buried.

Discussion

We intend to use the models of *P*2 myelin and CRBPI from our previous work to solve the structure of CRABP by molecular replacement. There are probably two molecules in the asymmetric units of both the $P4_1$ and $P2_1$ crystals. Attempts to solve the structure by molecular replacement have not been successful so far. We have tried two different program systems, *X-PLOR* (Brünger, 1990) and *AMORE* (Navaza, 1987, 1990; Castellano, Oliva & Navaza, 1992), six different search models, different resolution ranges and various data sets. In all cases we failed to obtain outstanding peaks in either the rotation function, the (combined) translation function, or both. Several times we arrived at false solutions which were rejected on the basis of packing, the absence of density for parts of the structure which were not included in the search model, or difficulties in simulated-annealing refinement. Therefore, we are currently screening for heavy-atom derivatives.

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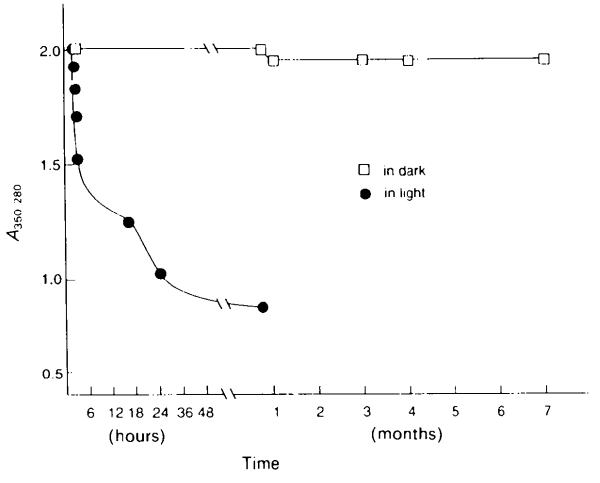

Fig. 1. Retinoic acid absorbs at 350 nm. The protein–retinoic acid spectrum can be evaluated by the ratio $A_{350\ 280}$. For protein fully saturated with retinoic acid, this ratio is 2.07. When protected from the light, the complex is stable for at least 7 months and this value remains essentially unchanged. When exposed to light, deterioration begins within 30 min, as monitored by a decrease in the absorbance of retinoic acid at A_{350}.

References

BANASZAK, L., WINTER, N., XU, Z., BERNLOHR, D. A., COWAN, S. & JONES, T. A. (1994). *Adv. Protein Chem.* In the press.

BRÜNGER, A. T. (1990). *X-PLOR: a System for Crystallography and NMR*. New Haven, CT: Yale Univ. Press.

CASTELLANO, E. E., OLIVA, G. & NAVAZA, J. (1992). *J. Appl. Cryst.* **25**, 281 284.

CHENG, L., QIAN, S.-J., ROTHSCHILD, C., D'AVIGNON, A., LEFKOWITH, J. B., GORDON, J. I. & LI, E. (1991). *J. Biol. Chem.* **266**, 24404 24412.

CHYTIL, F. & ONG, D. (1984). *The Retinoids*, Vol. 2, edited by M. B. SPORN, A. B. ROBERTS & D. S. GOODMAN, pp. 90–109. New York: Academic Press.

COWAN, S. W., NEWCOMER, M. E. & JONES, T. A. (1993). *J. Mol. Biol.* **230**, 1225–1246.

DOLLE, P., RUBERT, E., LEROY, P., MORRISS-KAY, G. & CHAMBON, P. (1990). *Development*, **110**, 1133–1151.

FIORELLA, P. D. & NAPOLI, J. L. (1991). *J. Biol. Chem.* **266**, 16572–16579.

FOGH, K., VOORHEES, J. J. & ÅSTRÖM, A. (1993). *Arch. Biochem. Biophys.* **300**, 751–755.

HARLOS, K. (1992). *J. Appl. Cryst.* **25**, 536–538.

JETTEN, A. M., ANDERSON, K., DEAS, M. A., KAGECHIKA, H., LOTAN, R., REARICK, J. I. & SHUDO, K. (1987). *Cancer Res.* **47**, 3523–3527.

JONES, T. A., BERGFORS, T., SEDZIK, J. & UNGE, T. (1988). *EMBO J.* **7**, 1597 1604.

KABSCH, W. (1988). *J. Appl. Cryst.* **21**, 916–924.

KEIDEL, S., RUPP, E. & SZARDENINGS, M. (1992). *Eur. J. Biochem.* **204**, 1141–1148.

LEVIN, M. S., LOCKE, B., YANG, N.-C. C., LI, E. & GORDON, J. I. (1988). *J. Biol. Chem.* **263**, 17715–17723.

LI, E., LOCKE, B., YANG, N. C., ONG, D. E. & GORDON, J. I. (1987). *J. Biol. Chem.* **262**, 13773–13779.

LI, E., QIAN, S.-J., WINTER, N. S., D'AVIGNON, A., LEVIN, M. S. & GORDON, J. I. (1991). *J. Biol. Chem.* **266**, 3622–3629.

LIU, Z.-P. (1993). PhD dissertation. Univ. of Texas, Southwestern Medical Center at Dallas, Texas, USA.

MCPHERSON, A. (1982). *Preparation and Analysis of Protein Crystals*. New York: John Wiley.

MADEN, M., ONG, D. E., SUMMERBELL, D. & CHYTIL, F. (1988). *Nature (London)*, **335**, 733–735.

NAVAZA, J. (1987). *Acta Cryst.* A**43**, 645–653.

NAVAZA, J. (1990). *Acta Cryst.* A**46**, 619–620.

NEWCOMER, M. E., JONES, T. A., ÅQVIST, J., SUNDELIN, J., ERIKSSON, U., RASK, L. & PETERSON, P. A. (1984). *EMBO J.* **3**, 1451–1454.

NEWCOMER, M. E., LILJAS, A., ERIKSSON, U., SUNDELIN, J., RASK, L. & PETERSON, P. A. (1981). *J. Biol. Chem.* **246**, 8162–8163.

NEWCOMER, M. E., LILJAS, A., SUNDELIN, J., RASK, L. & PETERSON, P. A. (1984). *J. Biol. Chem.* **259**, 5230–5231.

NINOMIYA, M., SUGANUMA, M., PAIK, N. S., MUTO, Y. & FUJIKI, H. (1988). *FEBS Lett.* **233**, 255–258.

RAGSDALE, C. W., PETKOVICH, M., GATES, P. B., CHAMBON, P. & BROCKES, J. P. (1989). *Nature (London)*, **341**, 654–657.

SATO, M., YAMAMOTO, M., IMADA, K., KATSUBE, Y., TANALIA, N. & HIGASHI, T. (1992). *J. Appl. Cryst.* **25**, 348–357.

STUMP, D. G., LLOYD, R. S. & CHYTIL, F. (1991). *J. Biol. Chem.* **266**, 4622–4630.

STURA, E. A. & WILSON, I. A. (1992). *Crystallization of Nucleic Acids and Proteins*, edited by A. DUCRUIX & R. GIEGÉ. New York: Oxford Univ. Press.

TABOR, S. & RICHARDSON, C. C. (1985). *Proc. Natl Acad. Sci. USA*, **82**, 1074–1078.

TAKAGI, K., SUGANUMA, M., KAGECHIKA, H., SHUDO, K., NINOMIYA, M., MUTO, Y. & FUJIKI, H. (1988). *J. Cancer Res. Clin. Oncol.* **114**, 221–224.

TAKASE, S., ONG, D. E. & CHYTIL, F. (1986). *Arch. Biochem. Biophys.* **247**, 328–334.

TAMURA, K., KAGECHIKA, H., HASHIMOTO, Y., SHUDO, K., OHSUGI, K. & IDE, H. (1990). *Cell Diff. Develop.* **32**, 17–26.

ULTSCH, M., DE VOS, A. M. & KOSSIAKOFF, A. A. (1991). *J. Mol. Biol.* **222**, 865–868.

UYEMURA, K., YOSHIMURA, K., SUZUKI, M. & KITAMURA, K. (1984). *Neurochem. Res.* **9**, 1509–1514.

WINTER, N. S., BRATT, J. M. & BANASZAK, L. J. (1993). *J. Mol. Biol.* **230**, 1247–1259.

ZELENT, A., KRUST, A., PETKOVICH, M., KASTNER, P. & CHAMBON, P. (1989). *Nature (London)*, **339**, 714–717.

ZHANG, J., LIU, Z.-P., JONES, T. A., GIERASCH, L. M. & SAMBROOK, J. F. (1992). *Proteins Struct. Funct. Genet.* **13**, 87–99.