Acta Cryst. (1996). D**52**, 203–206

Crystallization of a 14-3-3 protein.

BING XIAO,* DAVID JONES, JOEL MADRAZO,† YASMINA SONEJI, ALASTAIR AITKEN AND STEVE GAMBLIN *at Division of Protein Structure, National Institute for Medical Research, Mill Hill, London NW7 1AA, England*

(*Received 1 May 1995; accepted 12 July 1995*)

Abstract

Crystals of the tau (τ) isoform of the 14-3-3 family of proteins were grown and shown to belong to the orthorhombic space group $P2_12_12_1$ with cell dimensions $a = 70.29$, $b = 79.3$, $c = 101.00$ Å. The crystals were needle-like in morphology and less than 10 μm in two dimensions. Diffraction data were collected using synchrotron radiation sources from flash-cooled crystals. Native data extended to a resolution of 2.6 Å and mercury and platinum derivatives diffracted to 3.4 and 3.9 Å, respectively. The structure has been solved recently. Here the protein crystallization procedures, the characterization of the crystals and the correlation between crystal habit and diffraction quality are reported.

1. Introduction

The 14-3-3 family of proteins plays a critical role in a wide variety of cellular functions. Isoforms bind to and regulate the activity of Raf, protein kinase C and Bcr kinases, enzymes critical in signal transduction pathways. Amongst a host of other reported functions 14-3-3 isoforms are also implicated in exocytosis from adrenal chromaffin cells, regulation of the cell cycle in fission yeast, and transcriptional regulation in plants (reviewed in Aitken, 1995; Burbelo & Hall, 1995). There are five major mammalian brain forms of 14-3-3, named α–η after their respective elution positions on high-pressure liquid chromatography (Toker, Ellis, Sellers & Aitken, 1990; Ichimura *et al.*, 1988). The family is highly conserved and individual isoforms are either identical or contain a few conservative substitutions over a wide range of mammalian species. Isoforms have also been described from other mammalian tissues which are absent or present at low levels in brain. One particular isoform, tau (τ) 14-3-3 (245 residues, $M_r = 28$ kDa), was originally isolated from a human T-cell cDNA library (Nielsen, 1991). It associates with and is phosphorylated at serine by Bcr kinase (Reuther, Fu, Cripe, Collier & Prendergast, 1994). When expressed in *Escherichia coli* this isoform is a dimeric protein with identical secondary structure and biochemical characteristics to native 14-3-3 (Jones *et al.*, 1995) and its structure would be an excellent generic model for the different members of the 14-3-3 family. A site of phosphorylation has been described in some isoforms, and this may be an important regulatory feature. Thus, determination of the three-dimensional structure will help us to dissect the regulatory regions and sites of interaction with kinases and other proteins.

2. Experimental

Plasmid pKK233-2 (which produces a native protein expressed from a *trc* promoter; Pharmacia) was used as an expression vector for the production of τ 14-3-3. DNA manipulations and protein expression are described in Jones *et al.* (1995). *E. coli* TG1 (Sambrook, Fritsch & Maniatis, 1989) containing the overexpressing plasmid was grown overnight in 1 l L-broth plus 100 μg ml^{-1} penicillin, and this was used to inoculate 40 l of terrific broth in a bacterial fermenter. When the culture had reached OD = 1.0, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to 0.5 m*M*, and this was incubated for a further 4 h. This yielded 310 g wet weight cells which were resuspended in 20 m*M* Tris pH 7.5, 1 m*M* each ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA) and ethyleneglycol bis(β-amintoethyl ether) *N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 3 m*M* DL-dithiothreitol (DTT), 1 μg ml^{-1} each of leupeptin and pepstatin, 10 μg ml^{-1} soybean trypsin inhibitor, 1.56 mg ml^{-1} benzamidine and 75 μg ml^{-1} phenylmethyl sulfanyl fluoride (PMSF). Cells were disrupted by three passes through a French press, centrifuged at 10000 rev min^{-1} for 1 h in a JA14 rotor to remove insoluble material and stored at 193 K. Prior to purification, an aliquot of stored material was centrifuged for 1 h at 28000 rev min^{-1} in an SW28 rotor. The protein was purified on a Q-sepharose anion-exchange chromatography column and further purified by Mono Q chromatography (Jones *et al.*, 1995).

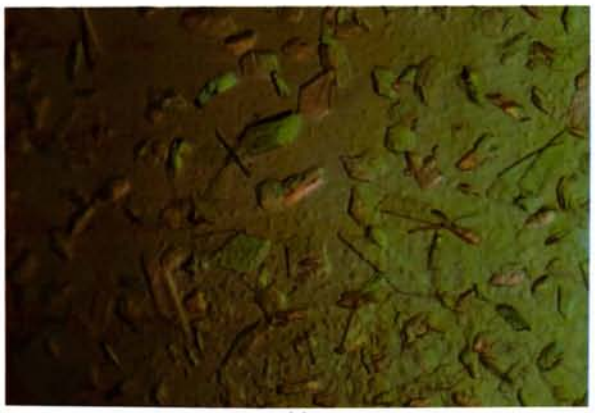
In initial experiments, crystals failed to grow using an established kit (Crystal Screen I, Hampton Research). After many trials, small prism-like crystals were grown from 20% PEG monomethyl ether 2 K, 15% saturated NaCl in 50 m*M* Tris pH 7.5 (protein concentration 6 mg ml^{-1}) in hanging-drops using the vapour-diffusion method at 277 K. The crystals diffracted to 3.3 Å at 9.6 SRS Daresbury, but crystal growth was not reproducable. It is possible that batch to batch variations in protein purity meant experiments were difficult to repeat. To avoid these batch effects, the recombinant protein was overexpressed in a 40 l bacterial fermenter so that further experiments gave constant results.

There are five free cysteines in a total of 245 amino residues in τ 14-3-3 which were thought to cause heterogeneity in the protein by oxidation even although the material was stored with 1 m*M* DTT. In an attempt to stabilize the protein solution, the cysteine residues were blocked by incubating for 1 h at room temperature in the presence of 25 m*M* iodoacetic acid, followed by gel-filtration purification. The carboxymethylated 14-3-3 samples were more easily crystallized than the unblocked sample. The solutions were monodisperse and dimeric as measured by light-scattering equipment DynaPro 801 (Protein Solutions, Ltd), with the protein concentration at 1 mg ml^{-1} in 1 m*M* EDTA, 1 m*M* EGTA, 1 m*M* DTT and 20 m*M* Tris pH 7.5. The solution recovered from light scattering was concentrated

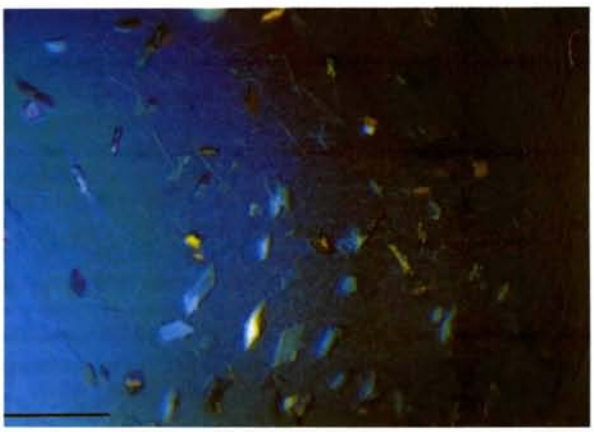
† Current address J. Madrazo, CIGB, Ave 31, Cubanacan, PO Box 6162, Havana, Cuba.

ISSN 0907-4449

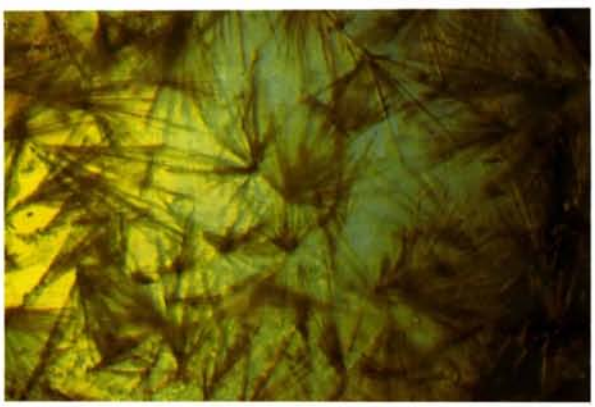
and screened using the Hampton Screen I. Carboxymethylated τ 14-3-3 formed crystals readily in a thin needle-like morphology, often in crystalline clusters, under five solution conditions of the kit. However, these were not stable at room temperature and dissolved overnight though some appeared again when the plates were placed at 277 K. Subsequent crystallization trays were always stored at 277 K. Several parameters were varied: salts such as ammonium phosphate, sodium chloride, potassium phosphate, lithium sulfate, magnesium chloride, calcium chloride; PEG with different molecular weights from 400 to 35 kDa; additives such as ethanol, acetone, dioxane, benzamidine hydrochloride, 1,2,3-heptanetriol; detergent such as 1-*O*-octyl-β-D-glucopyranoside (β-OG), *n*-dodecyl-*N*,*N*-ammoniopropyl sulfonate (SB-12), *N*,*N*-dimethyl-dodecylamine-*n*-oxide (C_{12}DAO); different buffers such as acetate, citrate, phosphate, cacodinate, MOPS, HEPES and MES at various pH levels from 4.4 to 9.0 in order to grow data-quality crystals.



(*a*)



(*b*)



(*c*)

Fig. 1. Crystals of τ 14-3-3. The bar in (*b*) represents 0.2 mm for all crystal scales in (*a*), (*b*) and (*c*). (*a*) Diamond-like crystals grown from a solution of 22% PEG 4 K, 2% saturated NaCl, 90 m*M* Li_2SO_4 in 80 m*M* MES at pH 5.9; (*b*) co-existing diamond and needle crystals grown from 23% PEG 4 K, 3% saturated NaCl, 135 m*M* Lu_2SO_4 in 80 m*M* at pH 6.2 and (*c*) needle-like crystals grown from 24% PEG 4 K, 4% saturated NaCl, 140 m*M* Li_2SO_4 in 80 m*M* MES pH 6.4.

Table 1. *The conditions causing the morphology change for τ 14-3-3*

		Diamond like	Diamond and needle co-exist	Needle like
PEG 4 K	[%(*w*/*v*)]	22	23	24
Saturated NaCl	(%)	2	3	4
Li_2SO_4	(m*M*)	90	135	140
80 m*M* MES	(pH)	5.8–6.0	6.1–6.2	6.3–6.7

The modified conditions for carboxymethylated τ 14-3-3 were then used to grow crystals of the unblocked τ (since the free cysteines were needed for binding heavy atoms to determine the phase in subsequent analysis). Crystals were grown in both hanging and sitting drops using the vapour-diffusion method from a solution of PEG 4K, NaCl, Li_2SO_4 in MES buffer over 7–10 d. The details of the conditions will be discussed later. Two different crystal morphologies were grown under quite similar conditions and indeed they even co-existed in the same drop; these are the diamond-like and needle-like crystals, shown in Fig. 1. Since the needle-like crystals were very small in two dimensions, initial efforts were focussed on growing bigger diamond-like crystals. It was apparent that the morphology of the crystals was very sensitive to the concentrations of PEG 4 K, NaCl, Li_2SO_4 and the pH in MES buffer (Table 1), other conditions were fixed as closely as

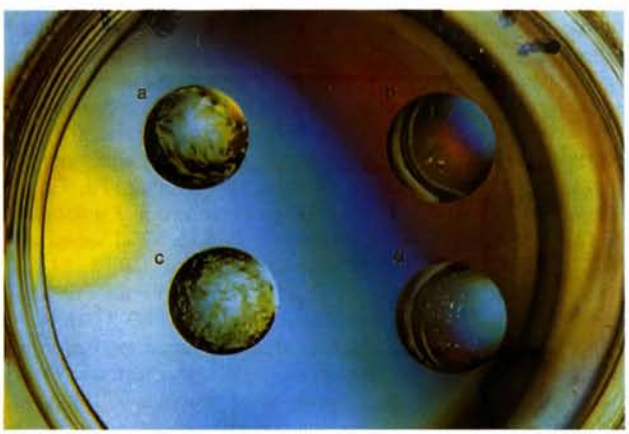


Fig. 2. Crystals of τ 14-3-3 in four drops on one single coverslip from four protein samples. (*a*) Fraction 1 of batch 1 – needle crystals; (*b*) fraction 2 of batch 1 – diamond and needle crystals; (*c*) fraction 1 of batch 2 – needle crystals; and (*d*) fraction 2 of batch 2 – diamond crystals.

possible. The two crystal morphologies were also seen to be a function of different fractions of the protein solutions collected from fast protein liquid chromatography, as shown in Fig. 2. Fig. 3 shows the sodium dodecyl sulfate polyacrylamide-gel electrophoresis gel of these four samples which are apparently identical except for a high molecular weight contaminant in the second fraction of batch 2 (Fig. 3*d*). It is not clear whether this contaminating protein caused the morphology change.

3. Results and discussion

The crystals were soaked in crystallization buffer with addition of 20% PEG 400. Two crystal forms have been tested at BL4/ID2 ESRF Grenoble, and 9.6 SRS Daresbury at 0.87 Å wavelength using a MAR research image plate. The crystals were mounted in rayon loops (Rodgers, 1994) and maintained at 100 K using an Oxford Cryosystem. The diamond crystals with maximum size 0.16 × 0.08 × 0.02 mm diffracted to 2.8 Å resolution. The diffraction data were processed using *DENZO* (Otwinowski, 1991) and showed that the crystal is monoclinic with cell dimensions *ca* $a = 124$, $b = 60$, $c = 144$ Å and $\beta = 106^\circ$. The diffraction pattern is shown in Fig. 4(*a*). However, none of the crystals had good order in the third dimension although more than 15 crystals were tried on the image plate. As a result, the needle crystals with maximum size 0.005 × 0.009 × 0.200 mm (which had been thought unsuitable) were tested and were found to diffract isotropically and strongly to at least 2.6 Å. The diffraction pattern is shown in Fig. 4(*b*). The crystal was grown from a solution of 24% PEG 4 K, 140 m*M* Lu_2SO_4, 4% of saturated NaCl in 80 m*M* MES at pH 6.4 (protein concentration 7 mg ml^{-1}); the space group was $P2_12_12_1$ and cell dimensions $a = 70.29$, $b = 79.30$ and $c = 101.00$ Å. A full set of data were collected from one needle crystal. The heavy-atom derivatives were similarly prepared in cryobuffer made up to 0.5 and 1.0 m*M* methyl mercury chloride and platinum terpyridine chloride respectively, soaking the needle crystals for 5–6 h. Complete data sets of Hg and Pt derivative were collected from needle crystals of similar size. However, the crystals were damaged by the heavy-atom compounds and became very fragile. The resolutions were, therefore, reduced to 3.4 and 3.9 Å. R_{iso} for the Hg derivative is 27.6 and for Pt is 22.5 ($R_{iso} = \sum_{hkl} |F_{der} - F_{nat}| / \sum_{hkl} F_{nat}$, where F_{nat} = protein structure-factor amplitude, F_{der} = heavy-atom derivative structure-factor amplitude), respectively.

We also prepared selenomethionyl crystals of τ 14-3-3 in order to solve the phase by the MAD (multiwavelength anomalous dispersion) method (Hendrickson, Horton & LeMaster, 1990) but the crystals were not well ordered.

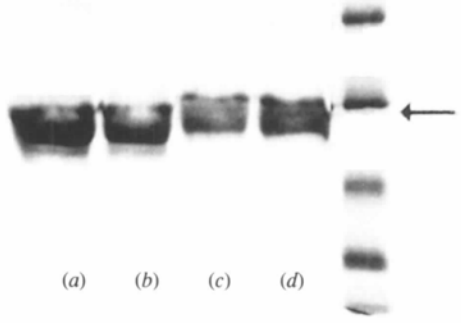


Fig. 3. SDS gel of these four protein samples in Fig. 2. The molecular weight marker at 30 kDa is arrowed. (*a*) Fraction 1 of batch 1, (*b*) fraction 2 of batch 1, (*c*) fraction 1 of batch 2 and (*d*) fraction 2 of batch 2.

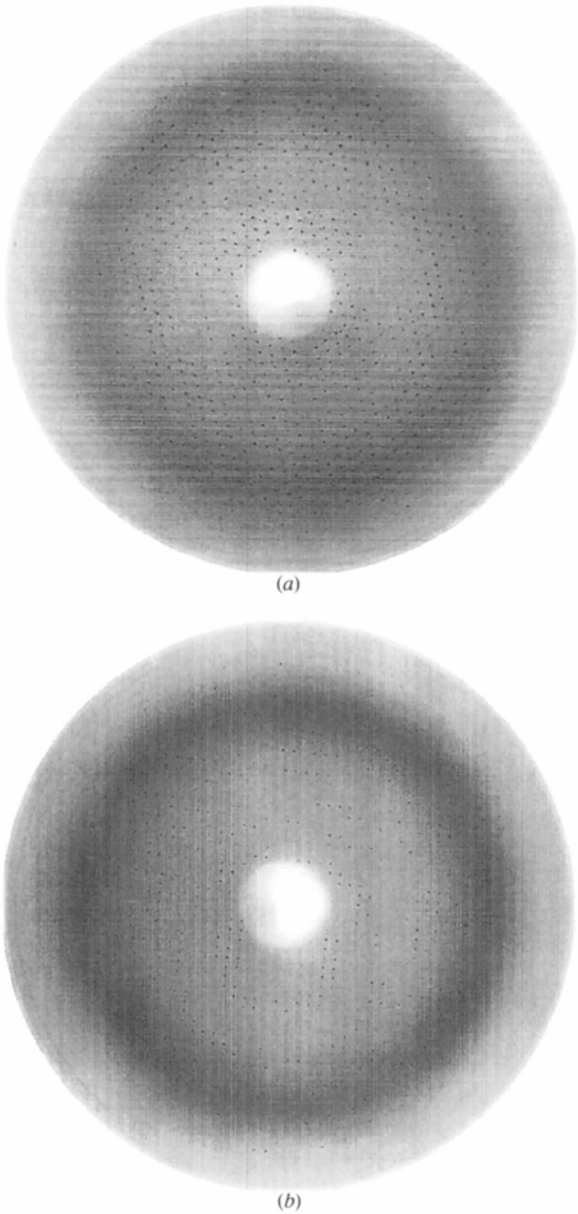


Fig. 4. 1° oscillation diffraction patterns were taken at BL4/ID2 ESRF Grenoble. The wavelength was 0.87 Å, the crystal-to-film distance was 400 nm and the resolution at the edge is 2.4 Å. (*a*) Diamond crystal, (*b*) needle crystal.

Although the needle crystals were of only modest size, the use of very powerful synchrotron sources and flash freezing of the crystals enabled us to readily determine the structure of the protein.

We are indebted to B. Rassmussen and C.-I. Brändén at ESRF Grenoble and P. Rizkallah and P. Lindley at SRS Daresbury for access to synchrotron facilities at short notice, Colin Young for the bacterial fermentation, Alan Harris for sequence analysis, and Ming He for assistance with crystallization.

References

Aitken, A. (1995). *Trends Biochem. Sci.* **20**, 95–97.
Burbelo, P. D. & Hall, A. (1995). *Curr. Biol.* **5**, 95–96.
Hendrickson, W. A., Horton, J. R. & LeMaster, D. M. (1990). *EMBO J.* **9**, 1665–1672.
Ichimura, T., Isobe, T., Okuyama, T., Takahashi, N., Araki, K., Kuwano, R. & Takahashi, Y. (1988). *Proc. Natl Acad. Sci. USA*, **85**, 7084–7088.
Jones, D., Martin, H., Robinson, K., Nielsen, P., Roseboom, P., Patel, Y., Madrazo, J. & Aitken, A. (1995). *J. Mol. Biol.* **245**, 375–384.
Nielsen, P. J. (1991). *Biochim. Biophys. Acta*, **1088**, 425–428.
Otwinowski, Z. (1991). In *Data Collection and Processing*, edited by L. Sawyer, N. Issacs & S. Bailey, pp. 56–62. Warrington, England: Daresbury Laboratory.
Reuther, G. W., Fu, H., Cripe, L. D., Collier, R. J. & Prendergast, A. M. (1994). *Science*, **266**, 129–133.
Rodgers, D. R. (1994). *Structure*, **2**, 1135–1140.
Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*, 2nd ed. Cold Spring Harbor Laboratory Press.
Toker, A., Ellis, C. A., Sellers, L. A. & Aitken, A. (1990). *Eur. J. Biochem.* **191**, 421–429.