Expression, purification and preliminary crystal analysis of the human low M_r phosphotyrosine protein phosphatase isoform 1

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Abstract The genes of the human low M_r phosphotyrosine protein phosphatase (PTPase) isoforms 1 (IF1) and 2 (IF2) were isolated by screening a human placenta cDNA library, cloned in pGEX and expressed in *E. coli* as fusion proteins with glutathione *S*-transferase. The recombinant proteins were purified by a rapid one-step procedure allowing each enzyme to purify with high final yield and specific activity. This result is important for IF1, whose purification from natural sources is difficult, due to precipitation propensity, thus hindering structural studies. The enzymes obtained showed kinetic parameters very similar to those previously determined for the enzymes purified by classical procedures from both human erythrocytes and rat liver. These recombinant enzymes can therefore be used in place of those purified from natural sources for every purpose. IF1 and IF2 crystals were also grown. IF1 crystals were X-ray-grade, diffracted to better than 2.4 Å and were suitable for high resolution X-ray structure determination.

Key words: Low molecular weight phosphotyrosine protein phosphatase; Low molecular weight phosphotyrosine protein phosphatase isoenzyme expression; Low molecular weight phosphotyrosine protein phosphatase isoenzyme purification; Low molecular weight phosphotyrosine protein phosphatase isoform 1/isoform 2 crystallization; Human low molecular weight phosphotyrosine protein phosphatase, recombinant

1. Introduction

Protein tyrosine phosphorylation/dephosphorylation plays an essential role in regulating cellular events such as proliferation, differentiation and oncogenesis in mammals [1–3]. These reactions are catalyzed by two classes of unrelated enzymes, protein tyrosine kinases and phosphotyrosine protein phosphatases (PTPases), respectively. In cells, the overall level of protein phosphorylation on tyrosine is therefore the result of a balance of the activity of these two classes of highly regulated enzymes [4].

At present, over fifty different PTPases are known; these are grouped into several families on the basis of catalytic and structural features (reviewed in [5]). The enzymes belonging to different families do not show any sequence similarity, but all share a common sequence CXXXXXRS/T (active site signature motif) containing some of the catalytic residues. In addition, catalysis of all PTPases proceeds through a cysteinyl-phosphate intermediate [6]. A group of PTPases comprises a number of highly homologous cytosolic enzymes formerly known as low M_r soluble acid phosphatases (E.C. 3.1.3.2) with molecular masses averaging 18 kDa. In mammals, these enzymes, presently known as low M_r PTPases (E.C. 3.1.3.48) (reviewed in [7]), are present as three couples of isoenzymes (A, B and C), each couple arising from alternative splicing of a specific allele [8,9]. The isoenzymes of each couple differ from each other only in the 40–73 region and are indicated as fast (f) or slow (s) form, respectively, on the basis of the electrophoretic mobility or, alternatively, as isoform 1 (IF1) and isoform 2 (IF2). Both isoforms hydrolyze phosphotyrosine, phosphotyrosine-containing peptides and proteins phosphorylated on tyrosine residues such as the activated EGF and PDGF receptors [10–13]; the enzyme is also able to interact with the stimulated insulin receptor [14] and its activity appears regulated in vitro and in vivo through phosphorylation by $pp60^{src}$ and by other kinases of the src family [15,16].

The X-ray crystal structure of the bovine liver IF2 has been determined at 2.1 Å resolution [17]. The enzyme is a closely packed structure formed by a central parallel, open twisted, four stranded β sheet surrounded on both sides by connecting helices. In the structure, the active site signature residues form a smooth loop (P-cradle) connecting the first α helix to the first β strand. The overall IF2 structure differs from that of other PTPases such as PTP1B, *Yersinia* PTPase and the dual specificity VHR [18–20], although all these enzymes possess the same P-cradle and similar active site conformations. Classical PTPases possess the active centre at the bottom of a crevice whose depth explains the inability of these enzymes to hydrolyze phosphoserine and phosphothreonine. In most PTPases, the crevice is lined by basic residues while in the case of the low M_r PTPases these are mainly hydrophobic and partly differ in IF1 and IF2. This feature can explain some of the differing catalytic properties of IF1 and IF2; moreover, it suggests that, in general, the residues lining the active site pocket can be considered one of the elements representing the molecular basis of the differential kinetic properties and substrate specificity of the varying PTPases [21].

Presently, a relevant number of information is available on IF2 structural, kinetic and biological features, whereas only the amino acid sequence and some kinetic data are known about IF1. The structural studies on IF1 have been hindered

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Abbreviations: IF1, low M_r PTPase isoform 1, corresponding to the A/B/C_{fast} nomenclature; IF2, low M_r PTPase isoform 2, corresponding to the A/B/C_{slow} nomenclature; PMSF, phenylmethylsulfonylchloride; EDTA, ethylenediamine tetraacetic acid; PNPP, *p*-nitrophenylphosphate; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; GST, glutathione *S*-transferase; UV, ultraviolet; PBS, phosphate-buffered saline; IPTG, isopropylthiogalactoside; PCR, polymerase chain reaction

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by the difficulty to obtain the enzyme, in a pure, active and native-like form and with a good yield, from its main natural sources (erythrocytes and rat liver) where it is present in low amounts. The description of the IF1 structural features could provide further information about the molecular basis of the differing kinetic behavior and substrate preference of either isoform. For these reasons, we isolated the cDNAs encoding human IF1 and IF2, respectively, and cloned them in the pGEX-2T expression vector in *E. coli* cells. Both isoforms were purified by a procedure allowing us to rapidly obtain high yields of each enzyme in a pure form. In addition, IF1 crystals suitable for X-ray analysis were obtained and their preliminary characterization is reported.

2. Materials and methods

E. coli Klenow fragment, DNA polymerase I, T4 DNA ligase, *Taq* DNA polymerase, polynucleotide kinase, random primer labelling kit and restriction enzymes were from Promega. Isopropylthiogalactoside (IPTG), pGEX-2T and oligonucleotide primers were from Pharmacia. [γ-^{32}P]-ATP (6000 Ci/mmol) and [α-^{32}P]-dATP (3000 Ci/mmol) were from New England Nuclear. The specific horseradish peroxidase-linked polyclonal anti-rat liver IF1 and IF2 antibodies used in the Western blot analysis were obtained as previously described [22]. The sparse matrix crystallization kits were Crystal Screen™ and Crystal Screen II™ from Hampton Research. Glutathione, cGMP, bovine thrombin and glutathione-agarose affinity gel were from Sigma. All other reagents were analytical grade or the best commercially available.

2.1. Oligonucleotide synthesis and enzyme cloning

The sequences of the two synthesized oligonucleotide primers (direct and reverse) were complementary to the conserved regions of the DNAs encoding either IF1 of the A phenotype or IF2 of the B phenotype. The direct primer ('Bat') sequence (5′-CCGCGTGGATCCGCGGAACAGGCTACC-3′) included the restriction site for *Bam*HI. The 3′-end oligonucleotide sequence was complementary to the sequences at the 5′ coding-ends of the cloned cDNAs. The ATG triplet coding for Met was deleted to get each enzyme with the appropriate N-terminus. The reverse primer ('Nat9') (5′-ACCGACCTGAGAAATGCAGGA-3′) previously used to sequence the same cDNA, was complementary to the cDNA 3′-untranslated sequence and did not contain any restriction site.

The template cDNA was obtained by screening a placenta cDNA library. The polymerase chain reaction (PCR) experiment was performed at 52°C under the conditions specified by the manufacturer, using 100 pmol of Bat and Nat9 primers, respectively. The fragment yielding the single 530-bp band, containing the complete IF1 and IF2 coding sequences (480 bp), was extracted from 1.5% agarose using Sephaglass™ BandPrep kit (Pharmacia) and treated with Klenow to generate blunt ends at both termini. The fragment was subcloned in the pGEX-2T vector after overnight digestion with *Bam*HI. The expression vector was prepared by digestion with *Eco*RI and treatment with Klenow to obtain the same blunt ends as the 530-bp fragment; the vector was also digested with *Bam*HI to produce the same site of the PCR-coding fragment and purified on 1.2% agarose gel. The recombinant expression vectors (pGEX-*IF1* and pGEX-*IF2*) were generated by ligating the digested vector and the PCR-coding fragment (*Bam*HI, blunt). The fragment was inserted downstream in an open reading frame with the GST sequence. The adequacy of the obtained cDNAs was checked by sequencing according to Sanger [23] (1977). Each construct was able to express the corresponding fusion protein and was used to transform TB1 *E. coli*.

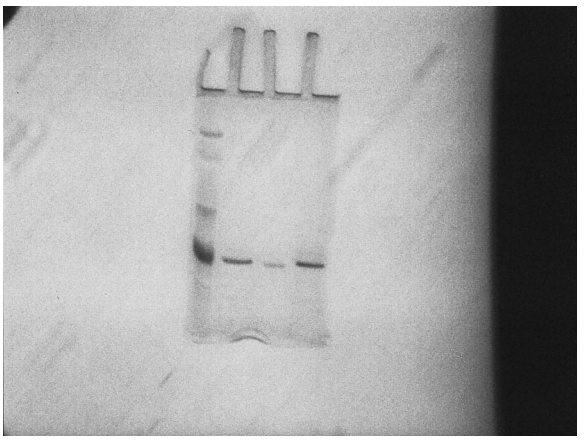

Fig. 1. Silver-stained 15% SDS-PAGE of purified IF1 (lane 2) and IF2 (lane 4). In lane 3 IF1 was run, extracted from the crystals after washing with the reservoir solution. Lane 1, standard proteins. From the top: bovine serum albumin (66 kDa), albumin (egg) (45 kDa), trypsinogen (bovine pancreas, PMSF) (24 kDa), β-lactoglobulin (subunit) (18.4 kDa).

2.2. Screening of expressing clones

The transformed *E. coli* cells were selected by ampicillin on agar plate. One pool of colonies was screened by restriction mapping using the *Bam*HI-*Pst*I restriction sites present in both the plasmid and the cloned cDNAs. Two bands of 1.0 and 0.450 kbp appeared, whereas only the 1.0-kbp band appeared when the wild-type plasmid was digested, thus demonstrating the presence and the correct orientation of the insert. The positive clones were checked for fusion protein expression. Clones were inoculated in 2 ml of LB medium supplemented with 100 μg/ml ampicillin. When cell suspension reached an optical density of 1.0, expression was induced by adding 0.2 mM IPTG (final concentration). After 4 h, the suspension was centrifuged and the pellet resuspended in 100 μl of 20 mM phosphate-buffered saline (PBS), pH 7.4, containing 150 mM NaCl, 10 mM 2-mercaptoethanol and 1.0 mM ethylenediamine tetraacetic acid (EDTA). The suspension was sonicated and centrifuged. The clear supernatant was checked for PTPase activity, run on 12% SDS-PAGE according to Laemmli [24] (1970) and subjected to Western blot analysis by using horseradish-conjugated anti-rat liver IF1 or IF2 antibodies, according to Modesti et al. [25].

Larger amounts of recombinant fusion protein were expressed under the conditions reported above by scaling up the procedure to 1 l or more growth medium. IPTG was added when the optical density of the suspension reached 1–2 units. After centrifugation, the bacterial pellet was stored at −20°C.

2.3. Protein determination and PTPase activity measurement

Protein concentration of the crude lysates was determined by Coomassie brilliant blue assay; in the case of the purified enzymes, protein concentration was determined by measuring UV absorption at 280 nm using an $A^{1\%}_{1cm} = 1.00$ or by amino acid analysis. Enzyme activity was measured using PNPP or L-phosphotyrosine as substrates as previously reported [21].

Table 1
IF1 and IF2 purification parameters[a]

Step	Volume (ml)	Total protein (mg)	Total activity (IU)	Purification (fold)	Yield (%)
Cell lysate (IF1)	150	200.0	2500	1.0	100.0
Cell lysate (IF2)	150	200.0	7000	1.0	100.0
Affinity (IF1)	20	25.0	1492	4.8	59.6
Affinity (IF2)	20	35.9	4378	3.5	62.5

[a]From a 2-l culture.

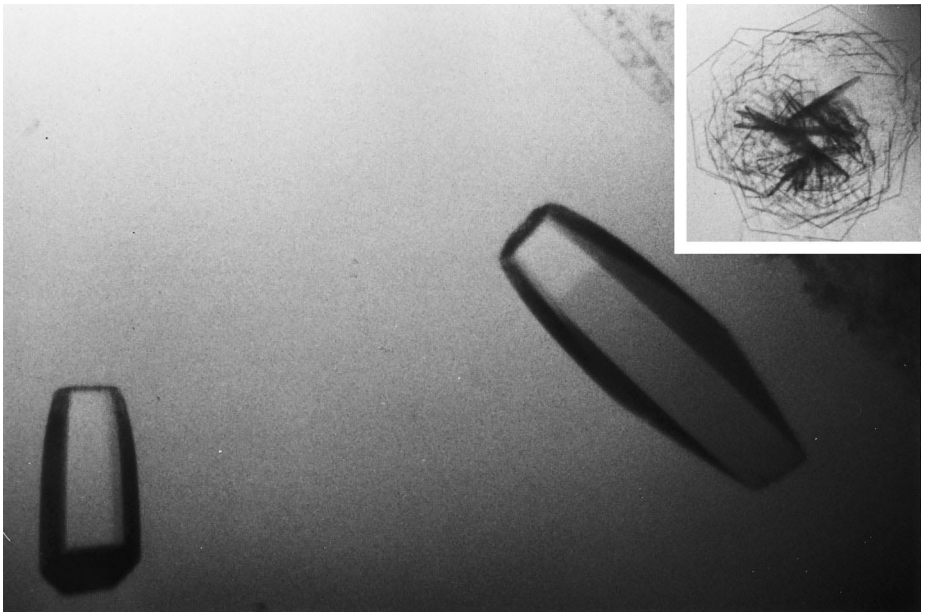

Fig. 2. IF1 and IF2 (inset) crystals grown as described in Section 2. Crystal and unit cell parameters are summarized in Table 3.

2.4. Amino acid analysis and mass spectrometry measurements

Amino acid analyses were performed using a Carlo Erba mod. 3A29 apparatus equipped with a computing integrator according to Manao et al. [26]. Values for serine and threonine were corrected for degradation during sample hydrolysis. The purified IF1 and IF2 ion spray mass spectra were obtained in positive ion mode and recorded on a Hewlett Packard model 1100 MSD mass spectrometer. Samples used for the determinations were dissolved in 50% acetonitrile/water containing 10 mM formic acid.

2.5. Enzyme crystallization and crystal analysis

Optimal crystallization conditions for both IF1 and IF2 were initially searched using the sparse matrix approach by the hanging drop vapor diffusion method in cell culture plates at room temperature and a protein concentration of about 5.0 mg/ml. The conditions yielding best crystals were then reproduced and optimized using a finer grid search. IF1 and IF2 crystals were grown in 3–4 days in drops of about 2 μl of protein solution to which was added 2 μl of reservoir solution (IF1: 40% saturation ammonium sulfate, 0.1 M sodium acetate, pH 5.6 and 10 mM dithiothreitol; IF2: 32–36% saturation ammonium sulfate, 0.1 M sodium acetate, pH 5.1 and 10 mM dithiothreitol). IF1 X-ray diffraction data were collected on a MAR-research imaging plate system mounted on a Siemens rotating anode or at the ESRF (Grenoble) at beam line BM2.

3. Results and discussion

IF1 (allele A) and IF2 (allele B) were expressed in *E. coli* as fusion proteins with glutathione *S*-transferase (see Section 2) and purified in milligram quantities using a one-step procedure performed by coupling the glutathione-agarose affinity chromatography to in-column overnight thrombin cleavage of the resin-bound IF1-GST or IF2-GST fusion protein, respectively. The frozen bacterial suspension in 20 mM PBS, pH 7.4, containing 1.0 mM EDTA, 10 mM 2-mercaptoethanol and 150 mM NaCl, was quickly brought to 37°C, then 1% Triton X-100 and 0.1 mM phenylmethylsulfonylchloride (PMSF) (final concentrations) were added; the suspension was treated with lysozyme (final concentration 1.0 mg/ml) for 30 min at room temperature and then sonicated 6 times for 90 s (overall). All subsequent operations were carried out at 5°C. The mixture was centrifuged at $12\,000 \times g$ for 20 min; the clear supernatant was diluted to a final protein concentration of 3–5 mg/ml with 20 mM PBS, pH 7.4, containing 1.0 mM EDTA, 0.1 mM PMSF, 10 mM 2-mercaptoethanol and 150 mM NaCl and assayed for PTPase activity. The supernatant was run at a flow rate of 50 ml/h through a 2.5-cm column filled with 20 ml of agarose-GSH resin (Sigma) equilibrated with 20 mM PBS, pH 7.4, containing 1.0 mM EDTA, 10 mM 2-mercaptoethanol and 250 mM NaCl. The column was washed with 15 volumes of the same buffer and equilibrated in 50 mM Tris-HCl buffer, pH 8.0, containing 150 mM NaCl (cleavage buffer). The fusion protein was cleaved by incubating overnight the resin bed in 10 ml of cleavage buffer

Table 2
Main kinetic parameters of purified human IF1 and IF2[a]

Parameter	IF1	IF2
Apparent K_m (mM)[b]	0.05 (0.06)	0.24 (0.42)
Apparent K_m (mM)[c]	0.34 (n.r.)	5.58 (n.r.)
k_{cat} (s^{-1})[b]	23.5 (9.9)	25.5 (14.7)
k_{cat} (s^{-1})[c]	20.3 (n.r.)	8.6 (n.r.)
k_{cat}/K_m (s^{-1} M^{-1} 10^3)[b]	470.0 (162.3)	106.4 (35.0)
k_{cat}/K_m (s^{-1} M^{-1} 10^3)[c]	59.8 (n.r.)	1.5 (n.r.)
Maximum cGMP activation (%)[b]	114.8 (n.r.)	470.0 (n.r.)

[a]In parentheses, the values calculated for enzymes purified from human erytrocytes [8]; n.r., not reported.
[b]Using PNPP as substrate.
[c]Using phosphotyrosine as substrate.

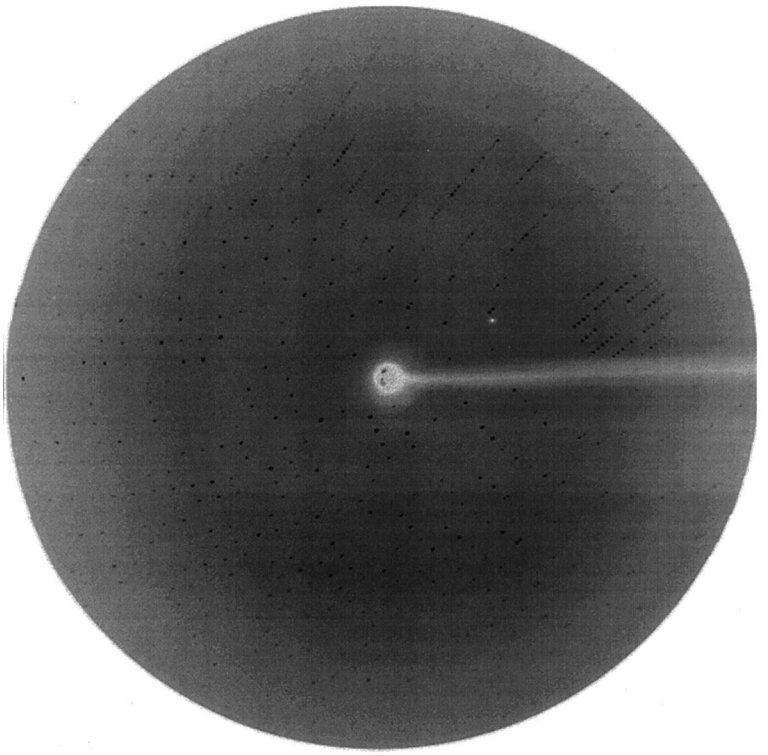

Fig. 3. X-ray diffractogram of IF1 crystals.

containing 4.0 mM $CaCl_2$ and thrombin at a 1:2000 ratio with respect to the estimated amount of fusion protein bound to the resin. At the end of the incubation, 0.1 volumes of 250 mM acetate buffer, pH 5.5, containing 2.0 M ammonium sulfate, 10 mM EDTA and 100 mM 2-mercaptoethanol (storage buffer) were added immediately, and the solution was further acidified to pH 6.0 by adding 0.1 volumes of 0.3 M acetic acid and assayed for protein content and PTPase activity. IF1 and IF2 specific activities, about 60 IU/mg and 120 IU/mg, respectively, were slightly higher than those reported for the same enzymes purified from natural sources. Finally, the enzyme was concentrated by ultrafiltration to about 10.0 mg/ml and its purity checked by SDS-PAGE, amino acid analysis and electrospray mass spectrometry. The SDS-PAGE (Fig. 1) of each purified enzyme showed an unique band whose molecular mass corresponded to those calculated for IF1 and IF2, respectively.

The amino acid analyses of the purified IF1 and IF2 were consistent with the known protein sequences, taking into account that both isoenzymes possess a Ser-Gly extension at the N-terminus due to the presence, in the fusion protein, of the thrombin cleavage site (PR-GS) between GST and PTPase. The molecular masses of the purified IF1 and IF2, as determined by electrospray mass spectrometry, were 18 082 and 17 992.8, respectively, in agreement with the values calculated from the amino acid sequences (IF1: 18 083.4 and IF2: 17 990.3), considering both the N-terminus extension and the lack of *N*-acetylation due to the expression of each fusion protein in a prokaryotic system.

The in-column thrombin cleavage of the GST-fusion proteins allowed more rapid purification of both isoforms and a higher protein cleavage efficiency. This purification procedure considerably reduced IF1 denaturation following thrombin cleavage, which is usually performed at more alkaline pH; in addition, the protein is maintained for most of the time at a concentration >3 mg/ml, thus preserving it from denaturation by dilution. Table 1 reports the IF1 and IF2 purification parameters, showing the good final yield of purified enzyme. In this way it is possible to obtain large amounts of both IF2 and, particularly, IF1, which is present in natural sources such as human erythrocytes and rat liver in quite low amounts and shows propensity to denaturate and precipitate when it is purified by conventional procedures.

Table 2 reports the main kinetic parameters of the purified recombinant IF1 and IF2. The apparent K_m values are very similar to those previously reported for the same enzymes purified from human erythrocytes [8], whereas the k_{cat} (and hence the k_{cat}/K_m) values are quite different. This apparent discrepancy can be explained taking into account that the values previously reported [8] were calculated at pH 6.0 instead of 5.5, a value closer to the pH optimum; in addition, as it has been pointed out, our purification procedure yields both enzymes, particularly IF1, mostly in the native, undenatured state, contrary to the classical purification procedure, thus

Table 3
Cell parameters of IF1 crystals

Space group (Å)	Cell (Å)	V_m (Å^3/Da)	Diffraction limit
$P6_522$	$a,b = 60.4$, $c = 167.8$	2.46	2.4

explaining the substantially higher specific activity value. Thus our results indicate that the lower (about 2/3) specific activity of IF1 with respect to IF2 reported by a number of authors is substantially attributable to the higher propensity to denaturation of the latter. A higher susceptibility to thermal denaturation of IF1 with respect to IF2 was previously reported [8]. The effect of cGMP on the purified recombinant enzymes is very similar to that found for the corresponding enzymes purified from rat liver [27,28]. In fact, only the IF2 catalytic behavior is affected by the presence of cGMP and the extent of activation is the same as that previously reported. These data indicate that the purified recombinant IF1 and IF2 maintain the same catalytic behavior as their natural counterparts and can therefore be used in substitution of these for every kind of kinetic as well as structural and biological study.

Recombinant IF1 and IF2 crystals were grown at room temperature as described in Section 2. The SDS-PAGE of IF1 redissolved from the crystals is shown in Fig. 1. Isomorphous IF1 crystals were reproducibly grown in 3–4 days in 40% saturation ammonium sulfate, pH 5.6, containing 0.1 M sodium acetate and 10 mM dithiothreitol. IF1 crystals belonging to space group $P6_522$ appeared as hexagonal prisms of typical dimensions $1.2\times0.2\times0.2$ mm (Fig. 2), with unit cell dimensions at room temperature of a, $b=60.4$ Å, $c=167.8$ Å and a V_m value of 2.46 Å^3/Da (Table 3), which is in the middle of the expected range and very similar to that reported for IF2 crystals previously obtained from the bovine liver enzyme [29]. These crystals diffracted to better than 2.4 Å on a rotating anode X-ray source and were well suited for high resolution crystallographic studies. Fig. 3 shows a diffractogram of an IF1 crystal obtained as indicated in Section 2.

IF2 crystals could also be grown under the conditions reported in Section 2. These small plate-like crystals (Fig. 2, inset) diffracted to 2.5 Å at beamline BM2 at the ESRF. However, the IF2 crystals have not yet been obtained as non-twinned single crystals, which did not allow further characterization. At any rate, our purpose was the achievement of good IF1 crystals useful for X-ray structural studies and, particularly, for enzyme structure determination.

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