**Protein Engineering** vol.10 no.12 pp.1461–1463, 1997

# Purification and crystallization of complexes modeling the active state of the fragile histidine triad protein

**Charles Brenner[1,2], Helen C.Pace[1], Preston N.Garrison[3], Angela K.Robinson[3], Angelika Rosler[4], Xiao-Hai Liu[4], G.Michael Blackburn[4], Carlo M.Croce[1], Kay Huebner[1], and Larry D.Barnes[3]**

[1]Kimmel Cancer Institute, Thomas Jefferson University, Philadelphia, PA 19107, [3]Department of Biochemistry, University of Texas Health Science Center, San Antonio, TX 78284, USA and [4]Department of Chemistry, Krebs Institute, University of Sheffield, Sheffield S3 7HF, UK

[2]To whom correspondence should be addressed

**Fragile histidine triad protein (Fhit) is a diadenosine triphosphate (ApppA) hydrolase encoded at the human chromosome 3 fragile site which is frequently disrupted in tumors. Reintroduction of *FHIT* coding sequences to cancer cell lines with *FHIT* deletions suppressed the ability of these cell lines to form tumors in nude mice even when the reintroduced *FHIT* gene had been mutated to allow ApppA binding but not hydrolysis. Because this suggested that the tumor suppressor activity of Fhit protein depends on substrate-dependent signaling rather than ApppA catabolism, we prepared two crystalline forms of Fhit protein that are expected to model its biologically active, substrate-bound state. Wild-type and the His96Asn forms of Fhit were overexpressed in *Escherichia coli*, purified to homogeneity and crystallized in the presence and absence of ApppA and an ApppA analog. Single crystals obtained by vapor diffusion against ammonium sulfate diffracted X-rays to beyond 2.75 Å resolution. High quality native synchrotron X-ray data were collected for an orthorhombic and a hexagonal crystal form.**

*Keywords*: Fhit/nucleotide-binding/tumor-suppressor/ApppA

## Introduction

The *FHIT* gene, located at the human chromosome 3 fragile site (Ohta *et al.*, 1996; Zimonjic *et al.*, 1997), encodes a dimeric polypeptide of 147 amino acids which cleaves diadenosine triphosphate (ApppA) to yield AMP plus ADP (Barnes *et al.*, 1996). Lesions in the *FHIT* gene are extremely common in tumors (Mao *et al.*, 1996; Mau *et al.*, 1996; Negrini *et al.*, 1996; Ohta *et al.*, 1996; Panagopoulos *et al.*, 1996; Shridhar *et al.*, 1996; Sozzi *et al.*, 1996a,b; Virgilio *et al.*, 1996; Yanagisawa *et al.*, 1996; Druck *et al.*, 1997; Gartenhaus, 1997; Geurts *et al.*, 1997; Shridhar *et al.*, 1997) and occur as early events in carcinogen exposed tissues (Sozzi *et al.*, 1996b). In a family predisposed to early-onset, multifocal renal carcinoma, a translocation that disrupts *FHIT* is transmitted to affected children; tumors from these individuals have lost both copies of *FHIT* (Ohta *et al*., 1996). Strikingly, in gastric, lung and kidney cancer cell lines that contain deletions of the *FHIT* gene, stable re-expression of Fhit protein suppressed the ability of the cell lines to form tumors in nude mice (Siprashvili *et al.*, 1997). The tumor suppressor activity of transfected *FHIT* genes was not lost when the codon for His96 was mutated to Asn (Siprashvili *et al.*, 1997) even though this mutation results in Fhit protein at least 1000-fold reduced for ApppA hydrolase activity (Barnes *et al.*, 1996). These observations suggest that *FHIT* is a tumor suppressor gene whose loss contributes to malignant transformation in multiple tissues. The ability to separate tumor suppressor function from hydrolysis suggests, as it had been hypothesized (Brenner *et al.*, 1997), that a substrate-bound form of the enzyme may be the biologically active state whose cellular function suppresses carcinogenesis.

Fhit-related proteins have been found in animals (Ohta *et al.*, 1996) and yeasts (Huang *et al.*, 1995; Brenner *et al.*, 1997) and constitute one branch of the histidine triad (HIT) superfamily (Seraphin, 1992) of nucleotide-binding proteins (Brenner *et al.*, 1997). An older branch of the HIT superfamily, the Histidine triad nucleotide-binding protein (Hint) branch, apparently predates the separation of bacteria, archaea and eukarya, having been found in all organisms (Brenner *et al.*, 1997). Crystal structures of nucleotide-bound forms of rabbit Hint demonstrated that the most conserved amino acids in the superfamily form an AMP or GMP binding site with the HIT motif, His-ϕ-His-ϕ-His-ϕ-ϕ (ϕ is a hydrophobic amino acid), constituting part of the α-phosphate binding site (Brenner *et al.*, 1997). Despite no overall sequence similarity, a monomer of *E.coli* galactose-1-phosphate uridylyltransferase (GalT) with its associated pyrimidine nucleotide (Wedekind *et al.*, 1995) can be superimposed upon the rabbit Hint dimer with its two bound purine nucleotides (Brenner *et al.*, 1997).

Recently, Lima and co-workers reported high resolution crystal structures of Fhit bound to adenosine (Lima *et al.*, 1997a), adenosine α/β-methylene diphosphate (AMP-CP), without adenosine bound, and in a form exposed to a mixture of adenosine and tungstate (Lima *et al.*, 1997b). These studies (Lima *et al.*, 1997a,b) demonstrated that Fhit possesses a GalT-half-barrel closely related to that of Hint with unique amino and C-terminal elaborations. Two identical adenosine nucleotide binding sites, as predicted from nucleotide-bound Hint structures (Brenner *et al.*, 1997), were described in the Fhit structure determinations (Lima *et al.*, 1997a,b).

Anticipating that the active, signaling state of Fhit protein may be bound to ApppA or AppppA substrates (Brenner *et al.*, 1997), and encouraged by the activity of the *FHIT* His96Asn allele as a tumor suppressor (Siprashvili *et al.*, 1997), we have undertaken a program to synthesize new hydrolysis-resistant analogs of this class of compounds (Blackburn *et al.*, 1998) for crystallographic analysis with wild-type enzyme. We have also undertaken a program to mutagenize active-site residues of Fhit (Barnes *et al.*, 1996) for crystallographic analysis with authentic ApppA. Here we report purification and crystallization of Fhit–diadenosine nucleotide complexes.

## Materials and methods

### *Overexpression, purification and crystallization*

The *FHIT* cDNA was cloned into pSGA02, transformed into *E.coli* strain SG100, and induced for 6 h at 30°C with 1 mM

**Table I.** Purification of Fhit

| Fraction | Protein (mg) | Activity (units[a]) | Specific activity (units/mg) | Yield (%) |
|---|---|---|---|---|
| Dialyzed crude supernatant | 993 | 8750 | 8.8 | 100 |
| DEAE-Sephacel | 129 | 9550 | 74.0 | 110 |
| Sephadex G75 | 62 | 3300 | 53.0 | 38 |
| QAE-Sepharose | 44 | 4700 | 107.0 | 54 |

[a]ApppA hydrolase activity of Fhit was assayed at 37°C with 100 μM [$^3$H]ApppA in 50 mM HEPES, pH 6.8, 0.5 $MnCl_2$.
One unit of activity was defined as the amount of enzyme that releases 1 μmol AMP per min under linear conditions.

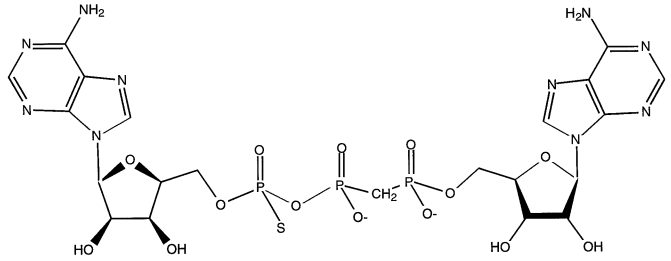

**Fig. 1.** Hydrolysis-resistant ApppA analog, IB2. The compound is a diadenosine triphosphate with $P^1$, $P^2$-methylene and $P^3$-thio modifications (Blackburn *et al.*, 1997). Only one stereoisomer at the phosphorothioate center is shown.

isopropyl-β-D-thiogalactoside (Ghosh and Lowenstein, 1997). A 52 ml crude supernatant was prepared from 14 g wet bacterial cell pellet by sonication in 42 ml buffer A [50 mM Na HEPES pH 6.8, 10% (vol/vol) glycerol] containing 0.5 mM phenylmethylsulfonyl fluoride, 1.1 μM leupeptin and 0.7 μM pepstatin. This material, dialyzed against buffer B [25 mM Na HEPES pH 6.8, 10% (vol/vol) glycerol] with 0.5 mM phenylmethylsulfonyl fluoride, was subjected to chromatography on a 210 ml column of DEAE-Sephacel (Pharmacia Biotech, Sweden) and eluted with an 800 ml gradient to 0.3 M NaCl in buffer B. Fractions containing ApppA hydrolase activity, assayed as described (Barnes *et al.*, 1996), were concentrated with a PM10 membrane (Amicon Corporation, Beverly, MA) and applied to a 180 ml column of Sephadex G75 superfine gel (Pharmacia) equilibrated with buffer A. Though the gel filtration step had only a one-third yield and resulted in no observable increase in specific activity, it was necessary to remove a peak of aggregated ApppA hydrolase activity from the major peak of activity which ran as expected of a Fhit dimer. The Fhit dimer peak was concentrated as above and chromatographed on a 21 ml column of QAE-Sepharose (Pharmacia). The homogeneous Fhit eluate from a 200 ml gradient to 0.2 M NaCl in buffer A was concentrated to 13.4 mg/ml (0.40 mM of dimer) in buffer A for crystallization. Purification data are summarized in Table I.

Fhit protein was mixed with an equal volume (2.5–4.0 μl) of 2 M ammonium sulfate, 4% polyethylene glycol 400, 0.1 M Na HEPES pH 7.5 on a siliconized cover slip which was sealed over a 0.8 ml volume of the same solution. Crystals grew at room temperature over the course of 1–4 weeks. Inhibited Fhit crystals were prepared by preincubating Fhit with 2.5 molar equivalents of IB2 (Blackburn *et al.*, 1997) (Figure 1) prior to vapor diffusion. Crystals grown in the absence of IB2 were typically orthorhombic prisms with dimensions as large as ~250 μm × ~250 μm × 1000 μm

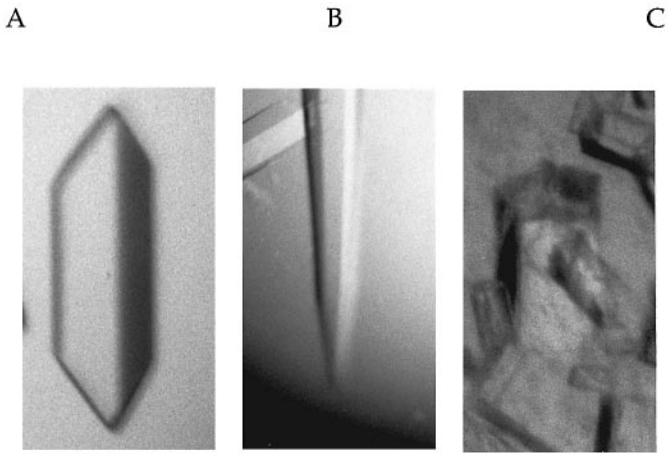


**Fig. 2.** Fhit crystals. Human Fhit protein, salted out with ammonium sulfate as described in Materials and methods, forms single orthorhombic prisms (**A**), single hexagonal needles (**B**), and pentagonal columns (**C**).

(Figure 2A). Inhibited crystals were usually hexagonal needles, ~150 μm wide by ~1000 μm in length (Figure 2B). Occasionally, crystals with pentagonal habits formed (Figure 2C) which diffracted X-rays to 2 Å resolution but did not appear to be single (data not shown).

Fhit His96Asn protein (Barnes *et al.*, 1996) was expressed, purified and crystallized as above except that hexagonal needles were obtained with IB2 and with authentic ApppA (Sigma).

*Synchrotron X-ray data collection*

Orthorhombic crystals were mounted in quartz capillaries and transported to beamline A1 (wavelength 0.908 Å, collimated to 150 μm) of the Cornell High Energy Synchrotron Source (CHESS). X-Ray diffraction data were collected on an ADSC charge coupled device (CCD) camera (Walter *et al.*, 1995), 185 mm from the crystal at 5 s per 1° oscillation. At CHESS beamline F1 (wavelength 0.918 Å, collimated to 150 μm), hexagonal crystals were washed in 2 M ammonium sulfate with 4% (vol/vol) polyethylene glycol 400 and 12% (vol/vol) glycerol, removed on nylon loops (Hampton Research, Laguna Hills, CA), and rapidly centered onto a goniometer head chilled by a liquid nitrogen cold stream. Data were collected on a Princeton 1k CCD, 225 mm from the crystal at 30 s per 1° oscillation. Data were indexed, integrated and averaged using the HKL package (HKL Research Inc., Charlottesville, VA).

## Results and discussion

Active, dimeric human Fhit protein was purified from *E.coli* with a high yield (Table I) and with a substantially higher specific activity than an affinity-purified Fhit fusion protein (Barnes *et al.*, 1996). Whereas the glutathione *S*-transferase–Fhit fusion protein had an apparent $k_{cat}$ value of ~3 $s^{-1}$ with ApppA as a substrate, the form of the protein purified in this study had a $k_{cat}$ value of at least 60 $s^{-1}$ and a $K_M$ value of 1.9 μM ($k_{cat}/K_M > 3.2 \times 10^7$ $M^{-1}$ $s^{-1}$). Moving from the affinity-purified fusion protein system to the current system has either increased the fraction of enzyme that is active, released an inhibitory fusion protein domain, or both. The preparation of Lima and co-workers (Lima *et al.*, 1997b) was only 2% as active as this preparation for reasons that are not apparent.

Purified Fhit crystallized in at least three discrete forms, two of which were suitable for X-ray diffraction analysis.

**Table II.** Crystallographic data

| Ligand | Space group | Temperature (°C) | Unit Cell lengths | | | Resolution (Å) | Reflections measured/unique | Completeness (%) | Rsym[a] (%) |
|---|---|---|---|---|---|---|---|---|---|
| | | | a | b | c | | | | |
| NONE | P2(1)2(1)2(1) | 20 | 51.96 | 149.56 | 219.20 | 50.0–2.75 | 177 630 / 39 117 | 85.8 | 5.8 |
| IB2 | P6(1)22 | –175 | 50.73 | 50.73 | 268.51 | 70.0–3.35 | 6201 / 2584 | 74.1 | 9.8 |
| | | | | | | 70.0–3.10 | 6659 / 2965 | 68.3 | 9.7 |

[a]Rsym = $\Sigma|I_{obs} - I_{avg}| \div \Sigma I$. $I$ = intensity.

Surprisingly, the orthorhombic form (Figure 2A) exhibited little decay in diffraction intensities in the synchrotron beam over the course of ~100° data collection sweeps. The hexagonal form (Figure 2B) was radiation sensitive at room temperature but, despite the presence of 2 M ammonium sulfate in the mother liquor, could be frozen in the cold stream simply by brief equilibration in 2 M ammonium sulfate, 4% (vol/vol) polyethylene glycol 400, 12% (vol/vol) glycerol. An additional crystal form had a pentagonal habit (Figure 2C) and diffracted well but could not be indexed as a single crystal.

Nearly complete data were collected for the wild-type orthorhombic form and the wild-type–IB2 complexed hexagonal form to 2.75 and 3.1 Å respectively (Table II). Because these crystals contain long cell lengths of ~220 and ~270 Å, data collection at home radiation sources is difficult. In order to separate reflections along the long axes of these crystal forms, X-ray detectors must be positioned at least 180 or 220 mm, respectively, from the crystals. The fine focus and high intensity of CHESS beamlines and the precise Cartesian resolution of CCD detectors were required for reliable diffraction intensities to be measured.

The orthorhombic form is of space group P2(1)2(1)2(1) and has a unit cell volume of 1 700 000 $Å^3$, consistent with a noncrystallographic multiplicity of 12 (six dimers) and a 42% solvent content (Matthews, 1968). The hexagonal form obeys the symmetry of space group P6(1)22 and its enantiomorph P6(5)22 and appears to be isomorphous with the P6(1)22 crystals of selenomethionyl Fhit (Lima *et al.*, 1997a). Crystal structures of the orthorhombic form of Fhit and the two diadenosine complexed forms modeling the active state of Fhit protein are in progress.

## Acknowledgments

This work was supported by NIH/NCI Grants CA75954 (to C.B.) and CA56336 (to C.M.C. for Kimmel Cancer Center core facilities), NSF Grant MCB-9604124 (to L.D.B.) and BBSRC Grant CHM.RA73861 (to G.M.B.). H.C.P. was supported by NIH/NCI Training Grant T32CA09662. CHESS is supported by NSF Grant DMR-9311772 and NIH Grant RR-01646. We thank Dan Thiel, the staff at CHESS, and Xiaofeng Zheng for assistance with data collection.

## References

Barnes,L.D., Garrison,P.N., Siprashvili,Z., Guranowski,A., Robinson,A.K., Ingram,S.W., Croce,C.M., Ohta,M. and Huebner,K. (1996) *Biochemistry*, **35**, 11529–11535.

Blackburn,G.M., Liu,X., Rosler,A. and Brenner,C. (1998) *Nucleosides* Nucleotides, **17**, in press.

Brenner,C., Garrison,P., Gilmour,J., Peisach,D., Ringe,D., Petsko,G.A. and Lowenstein,J.M. (1997) *Nature* Struct. *Biol.*, **4**, 231–238.

Druck,T., Hadaczek,P., Fu,T.B., Ohta,M., Siprashvili,Z., Baffa,R., Negrini,M., Kastury,K., Veronese,M.L., Rosen,D., Rothstein,J., McCue,P., Cotticelli,M.G., Inoue,H., Croce,C.M. and Huebner,K. (1997) *Cancer* Res., **57**, 504–512.

Gartenhaus,R.B. (1997) *Oncogene*, **14**, 375–378.

Geurts,J.M.W., Schoenmakers,E.F.P.M., Roijer,E., Stenman,G. and Van de Ven,W.J.M. (1997) *Cancer* Res., **57**, 13–17.

Ghosh,S. and Lowenstein,J.M. (1997) *Gene*, **176**, 249–255.

Huang,Y., Garrison,P.N. and Barnes,L.D. (1995) *Biochem.* J., **312**, 925–932.

Lima,C.D., DíAmico,K.L., Naday,I., Rosenbaum,G., Westbrook,E.M. and Hendrickson,W.A. (1997a) *Structure*, **5**, 763–774.

Lima,C.D., Klein,M.G. and Hendrickson,W.A. (1997b) *Science*, **278**, 286–290.

Mao,L., Fan,Y.H., Lotan,R. and Hong,W.K. (1996) *Cancer* Res., **56**, 5128–5131.

Matthews,B.W. (1968) *J. Mol. Biol.*, **33**, 491–497.

Mau,S., Ellis,I.O., Sibbering,M., Blamey,R.W. and Brook,J.D. (1996) *Cancer* Res., **56**, 5484–5489.

Negrini,M., Monaco,C., Vorechovsky,I., Ohta,M., Druck,T., Baffa,R., Huebner,K. and Croce,C.M. (1996) *Cancer* Res., **56**, 3173–3179.

Ohta,M., Inoue,H., Cotticelli,M.G., Kastury,K., Baffa,R., Palazzo,J., Siprashvili,Z., Mori,M., McCue,P., Druck,T., Croce,C.M. and Huebner,K. (1996) *Cell*, **84**, 587–597.

Panagopoulos,I., Pandis,N., Thelin,S., Petersson,C., Mertens,F., Borg,A., Kristoffersson,U., Mitelman,F. and Aman,P. (1996) *Cancer* Res., **56**, 4871–4875.

Seraphin,B. (1992) DNA Sequence, **3**, 177–179.

Shridhar,R., Shridhar,V., Wang,X.H., Paradee,W., Dugan,M., Sarkar,F., Wilke,C., Glover,T.W., Vaitkevicius,V.K. and Smith,D.I. (1996) *Cancer* Res., **56**, 4347–4350.

Shridhar,V., Wang,L., Rosati,R., Paradee,W., Shridhar,R., Mullins,C., Sakr,W., Grignon,D., Miller,O.J., Sun,Q.C., Petros,J. and Smith,D.I. (1997) *Oncogene*, **14**, 1269–1277.

Siprashvili,Z., Sozzi,G., Barnes,L.D., McCue,P., Robinson,A.K., Eryomin,V., Sard,L., Tagliabue,E., Greco,A., Fusetti,L., Schwartz,G., Pierotti,M.A., Croce,C.M. and Huebner,K. (1997) *Proc. Natl. Acad. Sci. USA*, **94**, 13771–13776.

Sozzi,G., Alder,H., Tornielli,S., Corletto,V., Baffa,R., Veronese,M.L., Negrini,M., Pilotti,S., Pierotti,M.A., Huebner,K. and Croce,C.M. (1996a) *Cancer* Res., **56**, 2472–2474.

Sozzi,G., Veronese,M.L., Negrini,M., Baffa,R., Cotticelli,M.G., Inoue,H., Tornielli,S., Pilotti,S., De Gregorio,L., Pastorini,U., Pierotti,M.A., Ohta,M., Huebner,K. and Croce,C.M. (1996b) *Cell*, **85**, 17–26.

Virgilio,L., Schuster,M., Gollin,S.M., Veronese,M.L., Ohta,M., Huebner,K. and Croce,C.M. (1996) *Proc. Natl Acad. Sci. USA*, **93**, 9770–9775.

Walter,R.L., Thiel,D.J., Barna,S.L., Tate,M.W., Wall,M.E., Eikenberry,E.F., Gruner,S.M. and Ealick,S.E. (1995) *Structure*, **3**, 835–844.

Wedekind,J.E., Frey,P.A. and Rayment,I. (1995) *Biochemistry*, **34**, 11049–11061.

Yanagisawa,K., Kondo,M., Osada,H., Uchida,K., Takagi,K., Masuda,A., Takahashi,T. and Takahashi,T. (1996) *Cancer* Res., **56**, 5579–5582.

Zimonjic,D.B., Druck,T., Ohta,M., Kastury,K., Croce,C.M., Popescu,N.C. and Huebner,K. (1997) *Cancer* Res., **57**, 1166–1170.

*Received May 29, 1997; revised October 19, 1997; accepted October 30, 1997*