Crystal Structure of Human Inosine Triphosphatase

SUBSTRATE BINDING AND IMPLICATION OF THE INOSINE TRIPHOSPHATASE DEFICIENCY MUTATION P32T*

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Inosine triphosphatase (ITPA) is a ubiquitous key regulator of cellular non-canonical nucleotide levels. It breaks down inosine and xanthine nucleotides generated by deamination of purine bases. Its enzymatic action prevents accumulation of ITP and reduces the risk of incorporation of potentially mutagenic inosine nucleotides into nucleic acids. Here we describe the crystal structure of human ITPA in complex with its prime substate ITP, as well as the apoenzyme at 2.8 and 1.1 Å, respectively. These structures show for the first time the site of substrate binding and imply the location of the substrate binding site has been postulated on the basis of weak binding of the non-physiological ATP analog, AMPPNP, to the enzyme (13). However, the mode of substrate binding proposed could not account for the strong binding preference for ITP, dITP, and xanthine triphosphates over the canonical nucleotides. Enzyme substrate interactions induce an extensive conformational change in the active site. We predict that the ITPA deficiency mutation P32T leads to a shift of this loop that results in a disturbed affinity for nucleotides and/or a reduced catalytic activity in both monomers of the physiological dimer.

Inosine monophosphate (IMP) is an essential metabolite in purine biosynthesis; it is the precursor of adenosine and guanosine monophosphates (AMP and GMP) (1). IMP is generated predominantly by de novo biosynthesis and by interconversion of nucleotide monophosphates. Inosine nucleotide triphosphates are present at low levels as byproducts generated either by deamination of purine bases or by phosphorylation of IMP. They are removed by the housekeeping enzyme inosine triphosphatase (ITP pyrophosphohydrolase; ITPase; ITPA; EC 3.6.1.19) that catalyzes the conversion of inosine triphosphate (ITP) to IMP and pyrophosphate (PPi). ITPase activity prevents accumulation of ITP and dITP, which may be incorporated into RNA and DNA, posing a risk for mutagenesis. ITPA orthologs are found in organisms from all kingdoms. In human and mouse, the protein is ubiquitously expressed (2, 3). Human ITPA is a 194-amino acid homodimer that is reliant upon Mg2+ ions for catalytic activity. ITP, dITP, and xanthine triphosphates are converted with similar kinetics, whereas the enzyme has very low affinity for other nucleotides. The pH optimum of ITPA is ~10 (2, 4, 5).

Genetic studies of individuals with detectable ITP levels in erythrocytes have linked ITPA deficiency to several single nucleotide polymorphisms in the ITPA gene. Of these, the mutation P32T abolished ITPase activity and caused accumulation of ITP in erythrocytes (6, 7). ITPase activity was lost in individuals homozygous for the P32T mutation, and it was reduced to ~25% in heterozygous subjects (8). This indicates that ITPase activity depends on the integrity of both protomers of the ITPA dimer. The ITPA P32T allele is present in all ethnic groups, being highest (11–19%) in Asian and lowest (1–2%) in Central and South American populations (7, 9–11).

ITPA deficiency is not linked to pathology in afflicted individuals, but perturbed ITP levels may be harmful under circumstances such as cellular stress. For instance, ITPA deficiency may be responsible for adverse drug reactions in patients treated with the purine analog azathioprine (12), an immunosuppressive drug used in the treatment of inflammatory bowel diseases, leukemia, and autoimmune complications in connection with organ transplants.

Crystal structures of several bacterial ITPA homologs have been solved in the absence of physiological ligands (13–15). The location of the substrate binding site has been postulated on the basis of weak binding of the non-physiological ATP analog, AMPPNP, to the Methanothermus enzyme (13). However, the mode of substrate binding proposed could not account for the strong binding preference for ITP, dITP, and xanthine triphosphate over other nucleotides (2, 14).

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The atomic coordinates and structure factors (code 2CAR and 2J4E) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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We have determined the crystal structure of human ITPA alone and in complex with one of its physiological substrates, ITP, and the products of hydrolysis, IMP + PPi. Our results identify the substrate and Mg2+ binding site of ITPA and show that it is different from the site previously proposed for this class of enzymes. Our structures provide an explanation for the high specificity of ITPA toward inosine and xanthine nucleotides. Comparison of the ITP-bound and unbound structures show that substrate binding induces closure of the nucleotide binding cleft and positions key side chains for catalysis. The structural implications of the P32T mutation causing ITPA deficiency are discussed.

EXPERIMENTAL PROCEDURES

Cloning and Protein Purification—Human ITPA cDNA (Mammalian Gene Collection clone 19624) was subcloned into pET28 (Novagen). Protein expression and purification were as described before (16) involving expression in Escherichia coli BL-21(DE-3) cells (Stratagene), cell lysis using a cell disruptor (Stansted Fluid Power), and perfusion on HisTrap HP columns (GE Healthcare). Eluted proteins were concentrated using Vivaspin cartridges (Millipore) to a volume of 1 ml and treated with 30 units of thrombin (GE Healthcare) overnight at room temperature. The protein sample was diluted 20-fold with 20 mM sodium citrate, pH 5.0, 20 mM NaCl, 10% glycerol, 1 mM Tris(2-carboxyethyl)phosphine and applied to a MonoS column (GE Healthcare). The protein passing the MonoS column was essentially pure ITPA liberated from the His6 tag as judged by SDS-PAGE and time-of-flight mass spectrometry analysis. ITPA was diluted 1:5 with 50 mM HEPES, pH 7.5, 300 mM NaCl, 10% glycerol, concentrated to 50 mg/ml, and stored as aliquots at −80 °C.

Protein Crystallization—Crystals were performed using a JCSG screen (17) with minor modifications. Monoclinic and trigonal crystals were obtained using the sitting drop method at 4 °C using 900 nl of protein (concentration 22 mg/ml) and 900 nl of well solution (0.2 M potassium chloride, 20% (w/v) polyethylene glycol 3350, pH 6.9). For the ITP complex crystals, the well solution contained 0.2 M potassium chloride, 31% (w/v) polyethylene glycol 3350, and the protein was added from a 50 mg/ml stock solution containing 10 mM ITP and 20 mM HEPES, pH 7.5.

Data Collection and Processing—Crystals were briefly soaked in a cryoprotectant (well solution containing 15–20% glycerol). Synchrotron diffraction data were collected at BESSY beamline BL-21 with 0.9 Å resolution by molecular replacement in Phaser/Phenix (21, 22) with structure 1B78 (“A hypothetical protein from Methanococcus jannaschii; Mj0226”) as model. After initial refinement the atomic resolution structure was solved using data from monoclinic crystals by molecular replacement in Molrep (23) using the partially refined structure as a model. After refinement in Refmac5 (24) and manual rebuilding, automated model building was carried out using ARP/wARP (25). Refinement was continued using SHEXL (26), applying the twinning operator (-h, k, l) and using intensities instead of amplitudes. The twinning fraction refined to 50%. In the final refinement rounds, anisotropic atomic displacement parameters were refined and hydrogen atoms were added. A total of 35 residues were built in two conformations, and the occupancies of the conformers were also refined in SHELX.

The ITP complex structure was solved by molecular replacement using the apo structure as a model and refined using Refmac5 with TLS parameters (27). Both structures were built using Coot (28). The complete protein chains were traceable; in the ITP complex the 122–128 loop was not built in all chains. Figures were made using PyMOL (www.pymol.org) and Ligplot (29). Superpositioning of structural models was performed using SSM (30). Domain motion analysis was carried out using DynDom (31).

RESULTS AND DISCUSSION

We have determined the crystal structure of human apoITPA at 1.1 Å resolution, as well as the structure of its complex with the physiological substrate (2.8 Å). ITPA with or without bound substrate crystallized as a homodimer, which is the physiological form of the protein (2). There were two and eight molecules/asymmetric unit in the apo and ITP complex crystals, respectively. Data collection, processing, and structure refinement statistics are summarized in Table 1.

Overall Structure—The structure of human ITPA consists of a central elongated mixed β-sheet forming a platform for two roughly globular lobes consisting of α/β structural elements (Fig. 1). NTP pyrophosphate hydrolases from bacteria have a similar architecture, such as M. jannaschii Mj0226 (PDB accession codes 1B78 and 2MJP) (13) and E. coli YggV (1VP2) (14). A superposition between the human apo ITPA structure (2CAR) and the Methanococcus enzyme (1B78) gives an root mean square deviation for Cα positions of 1.5 Å (184 aligned residues with 35% sequence identity).

The human ITPA structure shows, for the first time, how physiological substrate binds to an enzyme of this structural family: The two lobes from a single monomer form a substrate binding cleft between them. The dimer interface is made up mainly by interactions between the upper lobe of each monomer (the dimerization lobes; Fig. 1). The interface area between the monomers of the apo structure is 1080 Å2, with 9 hydrogen bonds and 9 bridging waters across the dimer interface.

Nucleotide Binding and Substrate Specificity—In the ITP complex structure (PDB accession code 2J4E), one molecule of ITP is bound in the cleft between the (upper) dimerization lobe and (lower) N-terminal lobe of the protein (Figs. 1 and 2). The purine base is wedged between the Phe-149 and Trp-151 side chains of the dimerization lobe so that their electron systems are stacked on top of each other. Phe-149 has flipped in toward the purine base compared with the apo structure. Three residues are within hydrogen bonding distance from the 6-keto oxygen of the inosine ring (Fig. 2A). Lys-172, His-177, and Arg-178. As these side chains point into the purine site from opposite directions, it is evident that the base is precisely positioned by this keto oxygen. Substrate specificity may be further enhanced by the hydrogen bonding network formed by these side chains and the Glu-22 carboxylate. An amino group
in the same position would interfere with the hydrogen bonding network, explaining the selectivity of the enzyme against adenine nucleotides (Fig. 2C). Xanthine nucleotides are also ITPA substrates (2). Additional specificity in xanthine binding may be obtained by a putative hydrogen bond between the 2-keto oxygen of the xanthine base and the Asp-152 backbone amide. The 2-keto oxygen binding pocket, however, is chemically not fit to accommodate an amino group, explaining the selectivity against guanine bases (Fig. 2C).

The ribose moiety is in a partly solvent-exposed pocket, and both ribose hydroxyls point into the solution without making strong contacts with the protein. This is consistent with the fact that 2-deoxyribonucleotides are also ITPA substrates (2). We also observed density consistent with a cation, presumably Mg$^{2+}$ as judged by its size and interactions, in the active site. The Mg$^{2+}$ ion and triphosphate tail make contacts with side chains from α-helices 2 and 3 and the central β-sheet (Fig. 2).

### TABLE 1

Summary of crystallographic data

The dataset obtained from trigonal crystals was not fully refined; see "Experimental Procedures" for a detailed explanation. R.m.s.d., root mean square deviation.

|               | Initial dataset | Apo | ITP complex |
|---------------|-----------------|-----|-------------|
| Beamline      | P2              | P2  | P1          |
| Wavelength (Å) | 0.9781          |     | 68.0, b = 75.3, c = 110.8; |
| Space group   | P3              |     |             |
| Unit cell dimensions (Å; degrees) | a = 50.0, b = 50.0, c = 309.2; | a = 31.2, b = 105.0, c = 50.1; | a = 85.1, β = 77.7, γ = 69.2 |
| Unique reflections | 39707          | 123899 | 45773 |

|                      | All data | High resolution shell | High resolution shell | High resolution shell |
|----------------------|----------|-----------------------|-----------------------|-----------------------|
| Resolution (Å)       | 20-2.25  | 2.95-2.25             | 40-1.09               | 1.20-1.09             |
| R$_{free}$ (%)       | 4.8      | 42.7                  | 4.4                   | 29.2                  |
| Completeness (%)     | 96.4     | 92.7                  | 92.5                  | 86.6                  |
| Redundancy (i/αl)    | 3.1      | 2.9                   | 4.6                   | 3.9                   |

**Ramachandran plot**

|                      | Most favored (%) | Additional allowed (%) | Generously allowed (%) | Disallowed (%) |
|----------------------|------------------|------------------------|------------------------|----------------|
| Protein              | 96               | 2.8                    | 0.6                    | 0.6            |
| Water                |                  | 5.3                    | 0.9                    | 0.6            |
| ITP and IMP          |                  |                        |                        | 0.6            |
| Mg$^{2+}$            |                  |                        |                        | 56.0           |

FIGURE 1. Dimeric structure of human ITPA. A, overview of the structure. Superposition of the apo (blue) and ITP-bound (pink) structures. The upper dimerization domain and the lower N-terminal domain form a cleft that closes upon substrate binding. Proline 32, the site of the ITPA deficiency mutation P32T, is indicated by an arrow for each monomer. B, detail of the electron density around residue Pro-32 in the apo ITPA structure (2Fo – Fe, contoured at 3σ). C, Fo – Fe, omit map of ITP and Mg$^{2+}$ in chain B (contoured at 3σ). D, Fo – Fe, omit map of IMP, pyrophosphate, and Mg$^{2+}$ in chain D (contoured at 4σ). C and D, the Glu-44 side chain is shown in stick representation.
Notably, all side chains discussed above are strictly conserved in the ITPA-like family of NTP pyrophosphatases (Fig. 3). The only exception is the eukaryotic Trp-151, which is a tyrosine in bacterial sequences; this is a conservative replacement in the sense that the tyrosine side chain can also stack with the nucleotide base.

Comparison of our ITPA structures shows a closure of the cleft by 25° upon ITP binding, brought about by displacement of structural elements from the N-terminal lobes (Figs. 1 and 3B). Two movements are most prominent: 1) An upward rigid body rotation of α-helix 2 around residues 11 and 26–32 (when the two structures are superimposed, the \( C_\alpha \) positions of residues 15–31 show a 4.8 Å root mean square difference between the apo and ITP-bound enzyme), and 2) rearrangement of β-strand 2 by bending around residues 39 and 44. This also results in a shift of α-helix 3. These changes upon substrate binding can be summarized as a movement of the N-terminal lobes toward the static dimerization lobes (Fig. 1). These rather drastic rearrangements may explain why crystals of bacterial NTPases cracked upon soaking of nucleotides (13, 14).

In the Mj0226 crystals (PDB accession code 2MJL), the non-hydrolyzable ATP analog AMPPNP was bound to one of the monomers at a site that differs from the ITP binding site seen in our human ITPA crystals (13). The differences are illustrated by superposition of the two structures with the respective nucleotides bound (Fig. 4). In the Mj0226 structure the adenine base of AMPPNP points away from the protein without interactions that explain specificity for the physiological substrates. In addition, the enzyme remains in the open cleft conformation similar to our apo structure, with which it also shows the best degree of superposition (described above). At the high concentration used, binding of the non-physiological ATP analog presumably took place at a secondary or
artificial site in the Methanococcus enzyme. We suggest that the substrate binding site revealed in our human ITPA structure, which has recently been predicted by Murzin and co-workers (15), is conserved across this enzyme family.

**Active Site and Putative Catalytic Mechanism**—In our ITPA crystals resulting from co-crystallization with the substrate ITP, seven of the eight molecules within the asymmetric unit had ITP bound (Fig. 1C). The putative Mg\(^{2+}\)/H\(_{11001}\) ion is in close proximity to the ITP \(/H_{9252}\)-and \(/H_{9253}\)-phosphates and is coordinated by the Glu-44 carboxylate. ITPA requires Mg\(^{2+}\)/H\(_{11001}\) or Mn\(^{2+}\)/H\(_{11001}\) for full activity and has a rather high Mg\(^{2+}\) optimum of 10 mM or more (2), suggesting involvement of an intermediate metal binding site. Although our ITPA protein substrate complex apparently contained endogenous metal ions through high affinity binding in the active site, additional metal ions were not added to the mother solution. Crystallization conditions were also suboptimal for catalysis with respect to pH and temperature. These factors may explain why we were able to crystallize the substrate complex. We assume that, under physiological conditions, Mg\(^{2+}\)/H\(_{11001}\) would bind at the site found in our structure.

In one of the protein chains (chain D) the density showed that the hydrolysis reaction had proceeded and IMP and PPi occupied the active site (Fig. 1D). The \(/H_{9251}\)- and \(/H_{9252}\)-phosphates had moved apart, and attempts to model in ITP resulted in negative density on the bridging oxygen. However, no major changes in nucleotide coordination resulted from this. The PPi phosphate group originating from the \(/H_{9253}\)-phosphate of ITP remains in the same position before and after catalysis (Fig. 2B). Nevertheless, the pattern of interactions suggests possible catalytic mechanisms. In the ITP-bound structure, the triphosphate tail of ITP is in the vicinity of side chains Asn-16, Lys-19, and Asp-41, whereas the cation is coordinated by the carboxylate of Glu-44 and the phosphates (Fig. 2A). A water molecule coordinated by the conserved Asp-72 would be ideally positioned for in-line nucleophilic attack on the \(\alpha\)-phosphate. Alternatively, the con-
erved Asn-16 could coordinate a water molecule for attack on the β-phosphate. A negatively charged transition state of the pyrophosphatase reaction might be stabilized by the terminal amino groups of the Lys-89 or Lys-19 side chains. In the D chain carrying IMP + PPi, the β-phosphate has moved by almost 0.5 Å toward the terminal amino group of Lys-89, supporting a role for Lys-89 as a stabilizer of the reaction intermediate.

Implication of the ITPA Deficiency Mutant P32T: Cross-talk between Monomers—The observation that individuals heterozygous for the P32T mutation retained 25% ITPase activity (9) suggested that both protomers of the physiological dimer need to be intact for catalytic activity. The ITPA dimer structure presented here suggests an allosteric regulatory mechanism by cross-talk between the protomers. The extended loop structure between β-strand 2 (residues 34–38) and the long α-helix 3 (residues 48–65) is positioned to transmit structural changes upon NTP binding and hydrolysis to the sister monomer. The Glu-46 backbone amide of each monomer forms a hydrogen bond with the Glu-44 backbone carbonyl oxygen of the sister monomer. Also the Gln-46 ε-amide of each monomer forms a hydrogen bond with the Tyr-45 backbone oxygen of the sister monomer. We suggest that NTP hydrolysis and/or nucleotide exchange in the two monomers are coupled across the dimer interface through these interactions. Dimer dissociation could explain the mutant effect; however, the affected loops constitute only a minor portion of the dimer interface. Rather, we think that the Asp-41 and Glu-44 carboxylates may act as triphosphate sensors, transmitting structural changes upon hydrolysis to the active site of the sister monomer, thereby regulating its activity.

The structures presented here suggest a mechanism for catalytic deficiency in P32T mutant ITPA. Comparison of our two structures suggests lobe movement and rearrangement of α-helix 2 as a mechanism for nucleotide exchange, as detailed above. Located in the hinge region of the flexible α-helix 2, Pro-32 is a key residue for positioning of side chains involved in substrate binding and catalysis. The adjacent extended loop structure between β-strand 2 and α-helix 3 contains residues of predicted catalytic involvement and is stabilized by interdimer contacts. Its position would likely be significantly disturbed by the P32T mutation. Thus, a disturbed nucleotide exchange or a partial misfolding of α-helix 2 would likely transmit across the dimer interface, rendering the dimer inactive.

Japanese populations feature the highest frequency (20%) of the P32T mutation. Interestingly, ITPA contains epitopes that are recognized by tumor-reactive cytotoxic T-lymphocytes in Japanese populations (32). Notably, these epitopes encompass the sequence that is likely to adopt a non-physiological conformation in the P32T mutant.

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