The kynurenine pathway has long been regarded as a valuable target for the treatment of several neurological disorders accompanied by unbalanced levels of metabolites along the catabolic cascade, kynurenine acid among them. The irreversible transamination of kynurenine is the sole source of kynurenine acid, and it is catalyzed by different isoenzymes of the 5′-pyridoxal phosphate-dependent kynurenine aminotransferase (KAT). The KAT-I isozyme has also been reported to possess β-lyase activity toward several sulfur- and selenium-conjugated molecules, leading to the proposal of a role in the enzyme in carcinogenesis associated with environmental pollutants. We solved the structure of human KAT-I in its 5′-pyridoxal phosphate and pyridoxamine phosphate forms and in complex with the competing substrate L-Phe. The enzyme active site revealed a striking crown of aromatic residues decorating the ligand binding pocket, which we propose as a major molecular determinant for substrate recognition. Ligand-induced conformational changes affecting Tyr181 and the Trp18-bearing α-helix H1 appear to play a central role in catalysis. Our data reveal a key structural role of Glu27, providing a molecular basis for the reported loss of enzymatic activity displayed by the equivalent Glu → Gly mutation in KAT-I of spontaneously hypertensive rats.

In mammals, the kynurenine pathway is the main route for the degradation of tryptophan exceeding anabolic needs and represents the source for de novo NAD biosynthesis. Several metabolites along this pathway, collectively identified as kynurenines, act as potent neuroactive compounds (1) exerting their function by either inducing free radicals generation (2) or engaging ionotropic excitatory amino acids receptors in the central nervous system (3). The amino acid L-kynurenine (L-Kyn)¹ is a key metabolite along this pathway, standing at the central branching point of the metabolic cascade (4). Indeed, L-Kyn undergoes different fates: i) it can be transformed to either 3-hydroxy-L-kynurenine or anthranilic acid and conveyed into the flux of freshly synthesized NAD, or ii) it can be used for the synthesis of kynurenine acid (KA). A major role in the central nervous system is ascribed to KA. In fact, it represents the only known endogenous antagonist of the excitatory action of excitatory amino acids, showing the highest affinities for the glycine modulatory site of the N-methyl-D-aspartate subtype of glutamate receptor (5–7) and the α7-nicotinic acetylcholine receptor (8–10). Its inhibitory action underlies its neuroprotective and neuroleptic properties; indeed, low endogenous brain KA level profoundly influences vulnerability to excitotoxic attacks (11–14). On the other hand, a KA increase significantly correlates with schizophrrenia (15) and cognitive impairment (16–18) suggesting an additional role of KA in the pathophysiology of psychiatric disorders and mental retardation.

The KA requirement in the central nervous system is satisfied by the in situ irreversible transamination of L-kynurenine, catalyzed by kynurenine aminotransferases (KATs) (Fig. 1). The catalyzed reaction proceeds first through the transaldimination between the L-Kyn α-amino group and the pyridoxal 5′-phosphate (PLP) cofactor following the classical mechanism reported for aminotransferases (19); however, the subsequent catalytic events leading to the kynurenic acid formation are far from fully elucidated. KATs belong to the α-family of PLP-dependent enzymes and are considered members of aminotransferases fold type I subfamily (20). So far two KAT isozymes, termed KAT-I and KAT-II, have been identified in rat (21–23) and human (24) brains and have been extensively characterized. Both isozymes are capable of catalyzing the transamination of L-Kyn and to a lesser extent 3-hydroxy-kynurenine to kynurenic acid and xanthurenic acid, respectively, using many different α-ketoacids as amino group acceptors (25, 26). KAT-I, also known as glutamine transaminase K, (27, 28) is strongly inhibited by the competing substrates glutamine, tryptophan, and phenylalanine (26). Of functional relevance, KAT-I also displays cysteine conjugate β-lyase activity (29) and has been shown to activate sulfur- and selenium-conjugated compounds (30, 31). This observation indicates an additional important role for KAT-I in the bio-activation of environmental pollutants contributing to liver- and kidney-associated carcinogenesis (28). Although the kidney and the liver show much greater KAT activity than the brain, the emphasis of KAT research has been placed on the enzymes in the central nervous system, paralleling the investigation of the pivotal role of KA therein.

We report here the crystal structure of human KAT-I (hKAT-I) at a resolution of 2.0 Å in its PLP and PMP forms as well as in complex with the competing substrate phenylalanine. Our results represent the first crystal structure of a human KAT to be reported, thus shedding more light into the

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catalytic mechanism and substrate specificity. The determination of the three-dimensional structure of hKAT-I may contribute to the rational design of selective inhibitors of high medical interest in a number of pathological conditions in humans.

**EXPERIMENTAL PROCEDURES**

**Enzyme Expression and Purification**

**Construction of Recombinant Transfer Vector for hKAT-I**—The open reading frame for hKAT-I (NP_872603) was amplified from human liver cDNA (Clontech) using a specific forward (5'-CGCAGTTGCGCCAAA-CAGCTGCGA, XhoI site underlined) and reverse (5'-AAAGCTTAGGTTG-TCACACTTCCAGTTT, Hind III site underlined) primers pair. The PCR product was inserted into a TOPO TA cloning vector and then subcloned into the baculovirus transfer vector pBlueBac4.5 (Invitrogen). The recombinant transfer vector (pBlueBac-HsKAT I) was sequenced for frame verification of the inserted DNA sequence.

**Production of Recombinant Baculoviruses for the Expression of hKAT-I in Insect Cells**—pBlueBac-HsKAT I was co-transfected with linearized Bac-N-Blue™ viral DNA (AcMNPV, Autographa californica multiple nuclear polyhedrosis virus), in the presence of InsectPlus™ insect cell-specific liposomes, to Spodoptera frugiperda (Sf9) insect cells (Invitrogen). The recombinant baculovirus was purified by plaque assay procedure. Blue putative recombinant plagues were transferred to 12-well microtitre plates and amplified in Sf9 cells. Viral DNA was isolated for PCR analysis to determine the purity of recombinant viruses. High titer viral stocks for individual recombinant viral stocks were generated by amplification in Sf9 cell suspension cultures according to the manufacturer’s instructions.

**hKAT-I Expression and Purification—Sf9** cells were cultured at 27 °C in an Ultimate Insect™ serum-free medium (Invitrogen) supplemented with 10 units of heparin/ml (Sigma) in spinner culture flasks with constant stirring at 80 rev/min. When the cell density reached 2 × 10⁶ cells/ml they were inoculated with the high titer viral stocks of the hKAT-I expressing recombinant baculovirus at a multiplicity of infection of six viral particles per cell.

At day 4 after hKAT-I recombinant baculovirus inoculation, Sf9-infected cells from a 2-liter culture were harvested by centrifugation (800 × g for 15 min at 4 °C). The pellet was dissolved in a lysis buffer containing 25 mM phosphate buffer, 150 mM NaCl, 0.1 mM PLP, 2 mM dithiothreitol, 2 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride with a final pH of 7.4. After a 30-min incubation on ice, cell lysate was centrifuged at 18,000 × g for 20 min at 4 °C, and the supernatant was collected and assayed for KAT-I activity. Soluble proteins in supernatant were precipitated with 65% saturated ammonium sulfate. Protein was collected and assayed for KAT-I activity. Soluble proteins in supernatant were precipitated with 65% saturated ammonium sulfate. Protein was collected and assayed for KAT-I activity. Soluble proteins in supernatant were precipitated with 65% saturated ammonium sulfate. Protein was collected and assayed for KAT-I activity. Soluble proteins in supernatant were precipitated with 65% saturated ammonium sulfate. Protein was collected and assayed for KAT-I activity. Soluble proteins in supernatant were precipitated with 65% saturated ammonium sulfate. Protein was collected and assayed for KAT-I activity. Soluble proteins in supernatant were precipitated with 65% saturated ammonium sulfate. Protein was collected and assayed for KAT-I activity.
RESULTS AND DISCUSSION

Overall Quality of the Model—The three-dimensional structure of the recombinant human kynurenine aminotransferase I has been solved by means of the molecular replacement technique at a resolution of 2.0 Å. A monomer is present in the asymmetric unit and the functional homodimer could be observed in the crystal lattice, resulting from the application of the crystallographic 2-fold axis. An excellent electron density map allowed the modeling of 415 residues of 422, one PLP, and 305 solvent molecules in the PLP form. The final model of the PMP form contains 415 residues, one PMP, and 197 solvent molecules, whereas the hKAT-I-Phe complex consists of 415 residues, one PLP molecule, one l-Phe molecule, and 202 solvents. The stereochemistry of the models has been assessed with the program PROCHECK (38). In the case of the PLP form, 90% of the residues were in the most favored regions of the Ramachandran plot. Pro123, Pro183, and Pro186 were all recognized as cis residues. Although Phe278 falls in a disallowed region of the Ramachandran plot in all three structures solved, the excellent electron density allowed us to unambiguously assign the observed conformation. All the figures have been generated with the programs MOLSCRIPT (39) and BOBSCRIPT (40).

Overall Structure—The hKAT-I protein architecture reveals the prototypical fold of aminotransferases subgroup I (19, 41), characterized by an N-terminal arm, a small domain, and a large domain (Fig. 2). The N-terminal arm consists of a random coiled stretch made of residues 4–17. The small domain (residues 18–43 and 302–421) folds in a 5-stranded mainly antiparallel β-sheet surrounded by five α-helices. The large domain (residues 44–301) shows a conserved α/β topology, where a sharply twisted 7-stranded β-sheet inner core is nested into a conserved array of eight α-helices contributed from both the interior and the exterior of the molecule.

As observed in other subgroup I aminotransferases, the functional unit of hKAT-I consists of a homodimer with subunits related by a dyad axis, with its two active sites located at the domain interface in each subunit, and at the subunit interface in the dimer (Fig. 3). Helices H2 and H13 participate in dimer stabilization as they are located around the 2-fold axis. Moreover, a relevant contribution toward the stability of the hKAT-I dimer is provided by its N-terminal arms. Mutational analysis carried out on pig cytosolic aspartate aminotransferase demonstrated that the integrity of the N-terminal region is of crucial importance for the enzymatic activity (42). In our crystallographic dimer, two strong salt bridges are established between Arg8 and Asp113 (at a distance of 3.5 Å) and between Arg8 and Glu114 (at a distance of 3.1 Å) of the opposite subunits; such interactions, while providing a major contribution to the dimer stability, lock the N-terminal arms in the observed conformation.

The PLP Binding Site—The active site contains one PLP molecule covalently linked to Lys247 and is hosted in a deep cleft at the domain interface built up by residues from both subunits (Fig. 3). Each PLP cofactor sits in a binding pocket defined by two regions contributed by residues of the large domains of both subunits (Fig. 4). The bottom of the PLP binding pocket is entirely defined by residues of the large domain of the corresponding monomer. With the exception of Lys247 and Gly100, all of these residues stand at or close to the edge of the inner core β-sheet, pointing toward the domain interface and facing the re-face of the PLP-ring. Distinct arrays of residues form the lateral walls of the PLP binding pocket. In particular, the stretch 34–37, together with the side chains of Arg398, Asn181, and Asn185 of the large domain, builds up one of the pocket walls. The opposite wall consists of residues Tyr101 and Tyr128 of the same monomer and of residues Phe278, His279, and Tyr636 of the other subunit. As a consequence, the PLP-pyridine ring can be seen as laterally clamped by a set of hydrophilic residues on one side and by numerous aromatic residues on the other one (Fig. 4).

In its PLP form, hKAT-I carries the PLP molecule covalently bound in the active site by a Schiff-base linkage to the catalytic Lys247 (Fig. 4A). This results in the presence of an internal aldime double bond (C4′=N) whose plane forms an angle of 123.0° with the pyridine ring of PLP. Several other residues contact the PLP molecule, participating in its recognition and binding. The phosphate group of the cofactor is engaged in a number of interactions with residues building up the strictly conserved “PLP-phosphate binding cup” featuring PLP-dependent enzymes (43). In particular, its OP1 oxygen makes a hydrogen bond with the Ser244 (2.6 Å), with its backbone nitrogen atom at 2.8 Å, and with the solvent molecule W7 (3.1 Å). The hydroxyl group of Tyr636 makes a hydrogen bond with the PLP OP2 atom (distance of 2.5 Å). A further hydrogen bond involves W34 located at 2.67 Å. Finally, the PLP OP3 atom makes interactions with Lys255 (at 2.7 Å from the NZ atom) and with the backbone nitrogen atoms of Gly100 and Tyr101 (distances of 3.2 Å and 2.9 Å, respectively). The PLP phenolic oxygen is held in place by its interactions with Tyr216 (at 2.7 Å from its OH atom) and with Asn185 (at a distance of 2.8 Å with its NH1 atom). Moreover, the N1 atom of the PLP pyridine moiety forms hydrogen bonds with the carboxyl oxygen atoms of Asp218. Several hydrophilic interactions participate in the further stabilization of PLP. In particular Phe125 and Val215 sandwich the pyridine moiety of the cofactor from its si-face and re-face, respectively.

Substrate Recognition and Implications for Catalysis—We have been unable to produce crystals of the hKAT-I in complex with the physiological substrate l-Kyn. Attempts at obtaining the structure of hKAT-l-Kyn complex invariably yielded crystals of the PMP form (Fig. 4B), emphasizing that our crystal form is catalytically competent. However, to describe the ligand binding site in hKAT-I, we solved the structure of the Michaelis complex with l-phenylalanine, which has been reported to inhibit hKAT-I by behaving as a very poor substrate and competing with l-Kyn for binding to the enzyme active site (26). In the hKAT-I-Phe structure (Fig. 4C), l-Phe lies just above the PLP molecule on its si-face. Several residues structurally conserved throughout aminotransferases described so far define the ligand binding site and contact l-Phe. In particular, Asp185 establishes a hydrogen bond with the ligand α-carboxylate moiety, which is further stabilized by Arg278. Such a conserved

| Data collection and refinement statistics | Native | PMP | l-Phe |
|------------------------------------------|--------|-----|-------|
| Resolution (Å)                           | 2.0    | 2.9 | 2.7   |
| Observations                             | 306,700 | 110,645 | 81,664 |
| Unique reflections                       | 54,768 | 18,243 | 22,371 |
| Rmerge                                  | 5.1    | 9.6  | 6.8   |
| Multiplicity                            | 5.6    | 6.1  | 3.7   |
| Completeness (%)                        | 99.9   | 99.3 | 98.3  |
| Refinement                               |        |      |       |
| No. of protein atoms                     | 3321   | 3321 | 3321  |
| No. of solvent molecules                 | 305    | 197  | 202   |
| No. of hetero atoms                      | 1.5    | 1.5  | 27    |
| Rwork (%)                               | 19.2   | 17.0 | 17.5  |
| R-free (%)                              | 22.7   | 22.0 | 23.0  |
| r.m.s.d. bond lengths (Å)                | 0.010  | 0.017| 0.020 |
| r.m.s.d. bond angles (°)                 | 1.17   | 1.66 | 1.85  |
| Mean B-factor main chain (Å²)            | 36.7   | 50.0 | 55.7  |
| Mean B-factor side chain (Å²)            | 35.7   | 51.8 | 57.4  |
| Mean B-factor hetero atoms (Å²)          | 28.6   | 31.6 | 55.5  |
| Mean B-factor solvent (Å²)               | 49.6   | 50.4 | 53.5  |

### TABLE I

| Human Kynurenine Aminotransferase I Crystal Structure | 50216 |
bonding scheme at the L-Phe carboxylic side results in the positioning of the substrate α-amino group just above the PLP C′ reactive center.

A remarkable structural trait of the ligand binding site in hKAT-I consists of a series of aromatic residues, including Tyr63*, His279*, Phe278*, Tyr101, Phe125, and Trp18, that is reminiscent of a crown (Fig. 4A). In the hKAT-I-Phe structure (Fig. 4C), L-Phe is sandwiched between Phe125 and His279*, in which the aromatic planes perpendicularly contact the ligand aromatic ring (distances for the closest pair of atoms being: 3.2 Å for L-Phe CE2-NE2 His279*, and 3.6 Å for L-Phe CE2–CZ Phe125). A further aromatic contact is provided by Phe278* (closest distance of 3.3 Å) oriented with its aromatic ring almost perpendicular to the ligand ring. Notably, Phe278* is the only residue falling in the disallowed region of the Ramachandran plot in hKAT-I structures, suggesting that the observed conformation is functional to substrate recognition and catalysis. A major question in kynurenine aminotransferases catalysis concerns the different substrate specificities of the isozyme. Understanding the molecular determinants resulting in L-Kyn recognition by hKAT-I will prove to be critical for the rational design of selective inhibitors of potential medical interest. Sequence alignment of subgroup I aminotransferases from different species revealed that a tyrosine residue peculiarly features human and rodent L-kynurenine aminotransferases (Tyr101 in hKAT-I). In hKAT-I we observed a striking conformational change affecting Tyr101. In fact, upon L-Phe binding, the Tyr101 side chain moves 3.5 Å away from its initial position, making room for the incoming ligand (Fig. 5). Being displaced from the crown of aromatic residues that decorates the L-Phe binding pocket, Tyr101 establishes a new hydrogen bond with Asn279 and becomes in contact with Cys127. Therefore, Tyr101 appears to assume two alternative conformations: a closed conformation in the PLP form and an open conformation in the liganded form of the enzyme. It has to be noted that Tyr101 has been observed in the closed conformation also in the structure of the PMP form of the enzyme (Fig. 4B). We therefore propose that Tyr101, by alternating between an open and a closed conformation along the catalytic cycle, acts as a gate controlling substrate admission to the enzyme active site.

In aminotransferases subgroup I, ligand binding induces conformational changes resulting in the closure of the enzyme active site (41, 44). Superimposition of the structures of hKAT-I in its PLP and phenylalanine-bound forms (Fig. 5) reveals a shift of the N-terminal α-helix H1 (residues Pro17–Glu27), which moves toward the active site upon substrate binding. As a result, Trp18 is brought into contact with the bound L-Phe, whose aromatic ring results perpendicularly oriented with respect to the Trp18 indole ring at a closest distance of 3.9 Å. Such an interaction appears to play an important role in ligand recognition and stabilization, completing the set of aromatic residues surrounding and contacting the ligand (Fig. 4C). In the recently reported structure of T. thermophilus glutamine: phenylpyruvate aminotransferase (ttGlnAT) (45), a bacterial...
ortholog of human KAT-I (46), an entire subdomain made of residues of the small domain, markedly moves to close up the active site upon ligand binding. However, the observed extension of the conformational change in hKAT-I is less pronounced than that in ttGlnAT upon 3-propionic acid binding (45), a molecule isosteric to L-Phe. A likely explanation could be that the Trp18 structurally equivalent position in ttGlnAT is occupied by the smaller amino acid phenylalanine (Phe15), which to contact the bound ligand, needs to move longer.

Because of our failure in obtaining the structure of hKAT-I in complex with the substrate L-Kyn, a crude model of the L-kynurenine Michaelis complex in hKAT-I was built by manually superimposing L-Kyn onto the ligand molecule in the hKAT-F1-Phe structure, minimizing for sterical collisions. Based on this model, we confirm the central role of the crown of aromatic residues in stabilizing the anthraniloyl moiety of L-kynurenine, and we suggest Trp18 as an important residue for substrate specificity. In fact, in our model a strong hydrogen bond is established between the benzyl amino group of L-kynurenine and the indolamine nitrogen atom of Trp18. However,
because our structural data clearly indicate a major role of the induced fit in ligand recognition, the description of the precise mode of binding of the substrate L-Kyn must await for the determination of the crystallographic structure of the hKAT-I-L-Kyn complex.

Role of Glu$^{27}$—Besides of its role as excitatory amino acids antagonist, in rodents KA is also involved in the control of the cardiovascular function by acting at rostral ventrolateral medulla of the central nervous system (47, 48). Spontaneously hypertensive rats, the most widely used animal model for studying genetic hypertension, have higher arterial blood pressure with respect to the normotensive Wistar Kyoto control rats. This defect has been associated with abnormally low KA levels in the central nervous system-specific districts tuning physiological blood pressure (49, 50). Sequencing analysis revealed the presence of a single point mutation in the KAT-I gene in all of the examined spontaneously hypertensive rats, leading to the substitution of a glutamate by a glycine at position 61 in the enzyme (51). Remarkably, the resulting mutated protein showed a severely reduced enzymatic activity, hinting at the key role of position 61 in catalysis (51). Sequence alignment of hKAT-I and rat KAT revealed the strict conservation of a glutamic residue in this position (Glu$^{27}$ in hKAT-I).

As described above, the conformational change affecting the helix H1 upon ligand binding is a key event in the catalysis. In hKAT-I, Glu$^{27}$ represents the C terminus of the helix H1 and appears as a pivotal residue in fixing the helix in the observed conformations. In fact, it makes a strong salt bridge with Lys$^{28}$ (distance of 3.5 Å), located in the center of the helix and a weaker interaction with His$^{26}$ (distance of 5.0 Å) located in the loop following the helix H1 (Fig. 6). Based on our structural data, we propose that the substitution Glu$^{61}$ → Gly observed in spontaneously hypertensive rats KAT (Glu$^{61}$ being equivalent to Glu$^{27}$ in hKAT-I) would affect the conformational change induced by substrate binding with dramatic consequences for the catalysis.

Conclusion—On the basis of our data, we propose that the observed conformational changes that occur in hKAT-I on ligand binding play a key role in catalysis. The shift of the α-helix H1 and the consequent recruitment of Trp$^{18}$ to the active site, together with the displacement of Tyr$^{101}$ (Fig. 6), result in a remodeling of the aromatic crown representing a major determinant for substrate binding. Whereas Trp$^{18}$ appears to play a relevant role in substrate specificity, Tyr$^{101}$ by alternating between its two conformations, controls substrate admission. Such structural information will provide a solid foundation for the rational design of small molecule inhibitors specifically targeting the hKAT-I isozyme, which will prove useful both as a tool for further investigation of the biological functions of KATs and as compounds of potential medical interest.

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Human Kynurenine Aminotransferase I Crystal Structure