Aromatic amino acid aminotransferase (ArATPh), which has a melting temperature of 120 °C, is one of the most thermostable aminotransferases yet to be discovered. The crystal structure of this aminotransferase from the hyperthermophilic archaeon Pyrococcus horikoshii was determined to a resolution of 2.1 Å. ArATPh has a homodimer structure in which each subunit is composed of two domains, in a manner similar to other well-characterized aminotransferases. By the least square fit after superposing on a mesophilic ArAT, the ArATPh molecule exhibits a large deviation of the main chain coordinates, three shortened α-helices, an elongated loop connecting two domains, and a long loop transformed from an α-helix, which are all factors that are likely to contribute to its hyperthermostability. The pyridine ring of the cofactor pyridoxal 5'-phosphate covalently binding to Lys235 is stacked parallel to F121 on one side and interacts with the geomemal dimethyl-CH/Y groups of Val201 on the other side. This tight stacking against the pyridine ring probably contributes to the hyperthermostability of ArATPh. Compared with other ArATs, ArATPh has a novel substrate specificity, the order of preference being Tyr > Phe > Glu > Trp > His >> Met > Leu > Asp > Asn. Its relatively weak activity against Asp is due to lack of an arginine residue corresponding to Arg292 (where the asterisk indicates that this is a residue supplied by the other subunit of the dimer) in pig cytosolic aspartate aminotransferase. The enzyme recognizes the aromatic substrate by hydrophilic interaction with aromatic rings (Phe121 and Tyr595) and probably recognizes acidic substrates by a hydrophilic interaction involving a hydrogen bond network with Thr264.

Aminotransferases have been widely applied in the large scale biosynthesis of unnatural amino acids, which are in increasing demand by the pharmaceutical industry for peptidomimetic and other single-enantiomer drugs (1). These enzymes have been classified into four families (I-IV) (2). Family I includes the aspartate aminotransferases (AspATs), aromatic amino acid aminotransferases (ArATs), alanine ATs, and histidinol phosphate aminotransferase. All members of Family I efficiently utilize α-ketoglutarate as an amino donor and glutamate as an amino acceptor. Eleven residues are invariant among the enzymes belonging to Family I (2). The members of Family I are further subdivided into three subfamilies according to their amino acid sequence alignment (2, 3). Subfamily Ia comprises AspATs isolated from Escherichia coli, yeast, chicken, pig, and other organisms, and ArATs from prokaryotes (E. coli and Paracoccus denitrificans). In this subfamily, an arginine residue (Arg292) according to the numbering for pig cytosolic AspAT (cAspATp) (4) is conserved. The Arg292 residue interacts with the ω-carboxyl moiety of the dicarboxyllic substrates (5-7). The arginine residue was not found in all members of subfamily Ia, despite the normally high degree of conservation in active site residues (2, 8, 9). Subfamily Ib is specialized for histidine biosynthesis (2, 4).

Recently, much research effort has been directed toward the isolation and characterization of enzymes from hyperthermophilic archaea. Interest in these enzymes has increased, because of their biotechnological potentials for novel application (10, 11) and because of the need for a better understanding of their intrinsic stability and denaturing processes. The mechanisms of their stability continue to be challenging and unresolved problems in biochemistry and biotechnology (10-13). An aspartate aminotransferase gene homolog (open reading frame identification number 1371) was identified via genome sequencing in the hyperthermophilic archaeon Pyrococcus horikoshii (14, 15). The gene (ArATPh) was expressed in E. coli, the product was purified to homogeneity, and the enzyme ArATPh was determined to be an aromatic aminotransferase belonging to subfamily Ia. We present the first report of the molecular structure of hyperthermophilic ArAT, which is an essential step in the effort to comprehend its stabilizing mechanisms. We also discuss its novel substrate specificity and dual substrate binding mechanism for both acidic and aromatic amino acids on the basis of its active site structure.
Materials and Methods

Chemicals—The pET-11a vector and ultracopolent E. coli XL2-Blue MRF* cell were purchased from Stratagene (La Jolla, CA). The pET-15b vector and E. coli strain BL21 (DE3) were obtained from Novagen (Madison, WI). Vent DNA polymerase was purchased from New England Biolabs (Beverly, MA). Restriction enzymes were purchased from Promega and Toyobo (Osaka, Japan) and were used according to the manufacturer’s recommendations. Utrapure dNTP solution was obtained from Amersham Pharmacia Biotech. L-Cysteine-insulinic acid, bovine DNase I, β-NADH, and malate dehydrogenase from porcine heart (mitochondrial) were purchased from Sigma. 2-Oxoglutaric acid monosodium salt and DTNB were purchased from Nacalai Tesque (Kyoto, Japan). Isopropyl-β-D-thiogalactopyranoside was purchased from TaKaRa Biotechnology (Shiga, Japan).

Cloning of Genes and Construction of Expression Vector—The complete gene sequence of P. horikoshii has been reported by Kavarabayasi et al. (14, 15). Standard cloning techniques were used throughout.

The aromatic aminotransferase (ArAT) gene was amplified using polymerase chain reaction with primers having NdeI and BamHI restriction sites according to a method reported previously (16). The sequences of the primers were 5'-TTTTTGCACTAATACATGCTGCTGAAGTGAGAAGCTA-3' (underlining indicates the upper primer containing the NdeI site) and 5'-TTTTTTGTAACCTTGGATCCTAACCAGGATT-AAAATGG-3' (underlining indicates the lower primer containing the BamHI site). The amplified gene was digested by NdeI and BamHI, and the digested fragment coding for ArAT was inserted in an expression vector pET-11a with the same restriction enzymes. The sequence of the inserted gene was verified by sequencing on an Applied Biosystems 373A DNA sequencer (Taq DyeDeoxy Terminator Cycle Sequencing Kit, Perkin-Elmer).

Overexpression and Purification of Recombinant Protein—The cloned gene was expressed using the pET-11a vector system in the host E. coli strain BL21 (DE3) according to the manufacturer’s instructions. The host cells were transformed with the constructed pET-11a/ArAT plasmid, after which the production of the protein was performed according to the method described previously (16). The concentration of the expressed protein was determined using a Coomasie protein assay reagent (Pierce) and utilizing bovine serum albumin as the standard protein. The crude enzyme solution was prepared from the transformant E. coli, and the enzyme was purified using chromatography in a HitTrap Q column (Amersham Pharmacia Biotech) and a HiLoad Superdex 200 column (Amersham Pharmacia Biotech) (16). The purity of the enzyme samples was analyzed using SDS-polyacrylamide gel electrophoresis (17) and isoelectric focusing using a PhastSystem (Amersham Pharmacia Biotech). Protein sequencing of recombinant ArAT was performed by Takara Shuzo Co. Ltd. (Osaka, Shiga, Japan) using a protein sequencer PSQ-1 (Shimazu, Japan).

Pre-steady-state Kinetic Studies of Half-transamination Reactions—Aliphatic amino acid substrates with unbranched side chains were used to estimate the hydrophobic substrate specificities of the aminotransferase. We used L-form amino acids for the sc3-c6 substrates and δ-isomers for the sc7-c9 substrates (18); the aminotransferase tested here cannot use γ-form amino acids as substrates. All measurements were carried out at pH 8.0 and 25 °C. The buffer solution contained 50 mM HEPES with 100 mM KCl, 20 mM EDTA, and various concentrations of L-aspartate or 2-oxoglutarate. The activity of the hydrophobic substrate phenylalanine in the pH 8.0 reaction mixture was monitored at 20°C using the molar extinction coefficient of 450 cm⁻¹ molar⁻¹ between phenylpyruvate and phenylalanine (22). The reaction mixture contained 50 mM HEPES, 100 mM KCl, 20 mM ArAT, and various concentrations of L-phenylalanine or 2-oxoglutarate.

To determine temperature dependence, the activities for five sets of substrates, tryptophan-2-ketoglutarate (Trp-2OG), tryptophan-phenylpyruvate (Trp-KetoPhe), histidine-2-ketoglutarate (His-2OG), histidine-phenylpyruvate (His-KetoPhe), and glutamate-phenylpyruvate (Glu-KetoPhe) were measured at different temperatures (25–90 °C) at pH 8.0. Based on the following molar extinction coefficients of 2-oxo acid derivatives, the concentration of the enzymatic products were calculated: at 310 nm, 24.5 and 3200 cm⁻¹ molar⁻¹ for 2OG and 3-indolepyruvate, respectively, and at 280 nm, 21 and 450 cm⁻¹ molar⁻¹ for 2OG and KetoPhe (22), respectively. The product from His was monitored at 293 nm using a coefficient difference of 3050 cm⁻¹ molar⁻¹ obtained to subtract the spectrum for the reaction of histidine with the PLP enzyme from that of the pyridoxamine 5'-phosphate enzyme (23). The reaction mixture contained 50 mM HEPES, 100 mM KCl, 20 mM ArAT, and various concentrations of amino acid derivatives.

Optimum Temperature and Thermostability for ArAT Reaction—The optimum temperature for ArAT activity was measured as described previously (24). The enzyme reaction was carried out in a solution (3.05 ml) containing ArAT (3.8 μM), l-cysteineinsulfonic acid (12.8 mM), 2-oxoglutaric acid (2.9 mM), EDTA (88 μM), and DTNB (1.5 mM) in 50 mM phosphate buffer (pH 6.5) at 30–98 °C, and the rate of increase in absorbance at 412 nm because of the reduction of DTNB was monitored for 5 min. For controls, the reactions were performed under the same conditions but without the enzyme.

To determine thermostability, the enzyme solutions (0.1 mg/ml) in 20 mM phosphate buffer (pH 6.5) were incubated at 95 °C for 90 and 120 min and then autoclaved in sealed Eppendorf tubes at 110 °C for 5, 15, 30, and 90 min. The heated enzymes were assayed in duplicate at 90 °C, as described elsewhere (24).

Spectroscopy of Coenzyme—To investigate the ionization of the internal Schiff base, the absorption spectra of the enzyme at a protein concentration of approximately 20 μM in a 1 cm cell were measured at 25 °C using a Hitachi spectrophotometer (model U-3000). The buffer solution was composed of 100 mM KCl, 0.91 mM EDTA, and a buffer component of 50 mM MES, 50 mM PIPES, or 50 mM HEPES.

pH Stability—The gross conformation and pH stability of ArAT were studied using CD spectroscopy. The CD spectra of ArAT, at a protein concentration of approximately 0.1 mg/ml in a 1-mm cell, were measured at 25 °C using a spectropolarimeter (J-720W, Jasco, Japan). The solution was comprised of 100 mM KCl and a buffer component of 50 mM MES, 20 mM phosphate, 50 mM carbonate, or 20 mM borate.

Scanning Calorimetry—The thermal denaturation curve of ArAT was measured using a Nano Differential Scanning Calorimeter (CSC5100, Calorimetry Science Co.). Before measurement, the enzyme solution (1 mg/ml) was dialyzed against 20 mM phosphate buffer, pH 6.5, and was degassed for 15 min using an aspirator. The sample cell was filled with the degassed enzyme solution, and the reference cell was filled with the outer solution from the dialysis. The measurement was performed at a temperature range of 0–125 °C. A scan rate of 1 K/min was used throughout. The denaturation profile was analyzed using Nano differential scanning calorimetry CpCalc data analysis software (Calorimetry Science Co.).

Structure Determination, Refinement, and Model Building—Crystals were obtained using the hanging drop vapor diffusion technique. An equi-volume of 3 μl 1.6-hexane-di-ol solution at pH 7.5 (100 mM HEPES buffer) containing 10 mM MgCl₂ was added to a protein solution containing 1.6% ArAT and 20 μM pyridoxal-5'-phosphate and a 10-μl droplet of the solution was equilibrated with 1 ml of a 3:1 1.6-hexane-di-ol solution. Crystals were grown at room temperature for 1 week. X-ray diffraction experiments were carried out on the Enraf FAST diffractometer equipped with a FRG517 image plate (ω + local spot size, 0.2 mm), and intensity data were collected at a resolution of 2.1 Å for the native crystal and at 3.0 Å for the heavy atom derivatives.

The structure was determined using the multiple isomorphous replacement method. A structure model was built on an electron density map calculated with multiple isomorphous replacement phases with a figure of merit of 0.93. The amino acid sequence was unambiguously

25 °C (20, 21), and the steady-state kinetics parameters, K, and kcat, were determined. The reaction mixtures contained 50 mM HEPES, 100 mM KCl, 0.01 mM EDTA, 0.1 mM NADH, 2.5 units/ml malate dehydrogenase, 1 μM ArAT, and various concentrations of L-aspartate or 2-oxoglutarate. The activity of the hydrophobic substrate phenylalanine in the pH 8.0 reaction mixture was monitored at 20°C using the molar extinction coefficient of 450 cm⁻¹ molar⁻¹ between phenylpyruvate and phenylalanine (22). The reaction mixture contained 50 mM HEPES, 100 mM KCl, 20 mM ArAT, and various concentrations of L-phenylalanine or 2-oxoglutarate.

Temperature Dependence of Activity for Overall Transamination Reaction—The overall transamination reaction for the acidic substrate aspartate was measured spectrophotometrically at 340 nm using a coupled assay with malate dehydrogenase and NADH at pH 8.0 and
Sequence Alignment and Phylogenetic Tree—We performed a sequence alignment of 11 aminotransferases within subfamily I by using the GeneWorks program (IntelliGenetics, Inc., Mountain View, CA) based on a PAM-250 scoring matrix. The compared enzymes were as follows: ArAT\textsubscript{Ph}, AspATs from thermophilic Bacillus sp. (38), Thermus thermophilus HB8 (8), Rhizobium meliloti (27), Bacillus subtilis (28), Methanococcus jannaschii (29), and Sulfolobus solfataricus (30); tryrosine ATs from human (31), rat (32), and Trypanosoma cruzi (33); and alanine ATs from human (34), rat (35), and Panicum milaceum (36). Phylogenetic trees for the same sequences were constructed using the GeneWorks program based on the unweighted pair group method with an arithmetic mean (37).

RESULTS

Sequence Alignment and Phylogenetic Tree of ArAT\textsubscript{Ph}—Because we were unable to construct a united alignment among aminotransferases belonging to the subfamilies 1α, 1β, and 1γ from P. horikoshii, other archaea, bacteria, and eukaryotes because of a lack of similarity between each subfamily, we made the alignment using 11 candidates belonging to subfamily 1γ to identify its conserved residues. The best alignment was obtained with the five thermophilic aminotransferases shown in Fig. 1. ArAT\textsubscript{Ph} showed poor identity to E. coli AspAT (AspATEc) (38), E. coli ArAT (ArATEc) (39, 40), and P. denitrificans ArAT (ArAT\textsubscript{Pd}) (41), which are well known members of subfamily Iα. According to these results, ArAT\textsubscript{Ph} was nominated to the aminotransferase subfamily Iγ (2, 3). Furthermore, ArAT\textsubscript{Ph} was closer to the thermophilic AspATs than to the tryrosine ATs from eukaryotes in subfamily 1γ.

Overexpression, Purification, and Oligomeric Structure of Recombinant ArAT\textsubscript{Ph}—The ArAT\textsubscript{Ph} gene was abundantly expressed in E. coli BL21 (DE3), and the recombinant ArAT\textsubscript{Ph} comprised 30% of the total protein. After heat treatment at 80 °C for 15 min, which removed most of the endogenous E. coli proteins, the protein was purified to homogeneity by sequential chromatography on HiTrap Q and HiLoad Superdex 200 columns. The final preparation of the ArAT\textsubscript{Ph} displayed a single band (42 kDa) on SDS-polyacrylamide gel electrophoresis. Isoelectric focusing indicated a pl value of 5.2 for ArAT\textsubscript{Ph}. The N-terminal sequence of the recombinant ArAT\textsubscript{Ph} was ALS-DRELEIVSEAEIRKL, which was identical to that deduced from the DNA sequence without the initial methionine residue. The enzyme had an apparent molecular mass of 56 kDa as estimated by gel filtration on a calibrated TSK gel G2000SWXL column, and a subunit molecular mass of 42 kDa as estimated by SDS-polyacrylamide gel electrophoresis. This suggests that it has a dimeric structure similar to other aminotransferases (24, 34, 42–45).

Optimum Temperature of the Recombinant ArAT\textsubscript{Ph}—The optimum temperature of this enzyme was 90 °C, which represents an extreme therophilic characteristic. The k\textsubscript{app} of ArAT\textsubscript{Ph} increased steadily in the range of the temperature studied here. The k\textsubscript{app} for l-cysteinsulfonic acid and 2-ketogulutaric acid as substrates was 1.39 × 10\textsuperscript{5} s\textsuperscript{-1} at 90 °C and pH 6.5. Like several other thermophilic enzymes (46–48), the recombinant ArAT\textsubscript{Ph} shows a thermal transition in conformation as indicated in Arrhenius plots near 70 °C (data not shown).

Substrate Specificity—ΔG\textsubscript{v} is the free energy difference between the unbound enzyme plus substrate (E + S) and the transition state (ES\textsuperscript{*}), was calculated for various substrates using Equation 1 or 2. A smaller ΔG\textsubscript{v} value indicates higher enzyme activity. As shown in Table I, Tyr is the best substrate having a k\textsuperscript{max}/K\textsubscript{d} (m\textsuperscript{-1}s\textsuperscript{-1}) value of 1.2 × 10\textsuperscript{5}. Three aromatic amino acids (Tyr, Phe, and Trp) and Glu are good substrates for ArAT\textsubscript{Ph}, whereas Asp is a poor substrate having a k\textsuperscript{max}/K\textsubscript{d} (m\textsuperscript{-1}s\textsuperscript{-1}) value of 9.1. ArAT\textsubscript{Ph} showed moderate activity on His. The activity of ArAT\textsubscript{Ph} toward a series of aliphatic substrates with straight side chains was enhanced as the side chain length increased. The activity of ArAT\textsubscript{Ph} was maximal for an 8-carbon substrate (2-amino octanoic acid).

Comparison of the Substrate Specificity for ArATs from Different Origins—The steady-state kinetic parameters of ArATs using an over expression of the reaction between 2OG and Asp or between 2OG and Phe were measured at 25 °C (the upper section of Table II). The enzyme activity against Phe was high with a k\textsuperscript{cat}/K\textsubscript{d} value of 5.2 × 10\textsuperscript{3} m\textsuperscript{-1}s\textsuperscript{-1}, but the activity against Asp was very low (2.2 m\textsuperscript{-1}s\textsuperscript{-1}). The difference between these k\textsuperscript{cat}/K\textsubscript{d} values was on the order of 10\textsuperscript{3}, whereas the difference for ArATEc (from E. coli) and ArAT\textsubscript{Pd} (from P. denitrificans) was approximately 10-fold. At the optimum reaction temperature of 90 °C, ArAT\textsubscript{Ph} has approximately a 10\textsuperscript{2}-
and 10-fold higher activity for His and Glu, respectively, than does ArATEc or ArATPd at 25 °C (Table II). This indicates that Glu is the best acidic substrate for ArATPd.

Spectroscopic Properties of the Enzyme-bound Coenzymes
PLP and Pyridoxamine 5'-Phosphate—An internal Schiff base is formed between Lys323 (corresponding to Lys358 in cAspATp) and the aldehyde group of the coenzyme PLP. The reaction produces a spectral change in the visible absorption region. Changes in the apparent molar absorption coefficients for the PLP form enzyme at 420 and 370 nm were plotted against pH.

The reaction temperatures are shown in parentheses.

| Substrate | ArATPd (25 °C) | ArATPc (25 °C) | ArATPd (25 °C) |
|-----------|----------------|----------------|----------------|
| Asp       | 2.2 ± 0.1      | 3.7 × 10^4     | 9.4 × 10^4     |
| Phe       | 2.0 ± 0.0 × 10^3 | 9.6 × 10^5     | 2.2 × 10^6     |
| Trp       | 6.3 ± 0 × 10^4 | 1.3 × 10^5     | 6.0 × 10^6     |
| His       | 5.5 ± 0.0 × 10^4 | 1.5 × 10^5     | 1.0 × 10^6     |
| Glu       | 4.2 ± 0.0 × 10^3 | 6.7 × 10^5     | 3.4 × 10^6     |
| 2-Ketoglutarate | 4.8 ± 0.0 × 10^3 | 1.7 × 10^5     | 7.1 × 10^6     |
| Phenylpyruvate | 1.1 ± 0.0 × 10^4 | 2.0 × 10^7     | 1.6 × 10^6     |

* Cited from Ref. 39.
* Cited from Ref. 41.
* The values were obtained using the kinetic parameters for the half-reactions.

and 10-fold higher activity for His and Glu, respectively, than does ArATEc or ArATPd at 25 °C (Table II). This indicates that Glu is the best acidic substrate for ArATPd.

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Three-dimensional Structure of ArATPc—The space group of the protein crystal is P2_12_12, and the cell dimensions are a = 64.01, b = 124.87, and c = 128.78 Å. The structure was solved using the multiple isomorphous replacement method at a resolution of 3.0 Å using four heavy atom derivatives: K_4P_tCl_6, methyl mercury chloride, p-chloromercuribenzenesulfonic acid, and mersalyl acid. These data are presented in Table III. The structure was refined at 2.1 Å resolution to the R value of 0.185 and R_free of 0.254, respectively. The root mean square deviations of bond distances and angles from their ideal values were 0.017 Å and 3.24°, respectively. The (α, β) values for all the amino acid residues except Thr264 fell in a normal region in the Ramachandran plot (data not shown). The crystal having a V_m = 2.9 Å³/Da contains two molecules related with local 2-fold symmetry in an asymmetric unit. ArATPc has a dimer structure (Fig. 2). One dimer molecule has two active sites, and each active site binds one PLP. In both subunits, the N-terminal region of residues 2–11 form a short α-helix, but region 12–26 is missing in the final structure model because no significant electron density was observed in the 2F_o – F_c and F_c – F_o maps for the region (Fig. 3). The molecule consists of two domains. The large domain has a α/β structure comprised of six α-helices (H3–H8) and seven β-strands (S1–S7), as assigned by the program DSSP (49). The strands form a twisted sheet structure, on both sides of which helices are arranged. The small domain consists of three α-helices (H10–H12) and a β-strand (S8). A long α-helix (H10) links to the large domain via an α-helix (H9).

The molecular replacement method using the structures AspATEc, ArATPd, and cAspATp as templates (6, 7, 50) was not successful for solving the ArATPc structure because of poor identity of the primary sequences and a large deviation in the main chain coordinates. By the least square fit after superposing ArATPc on ArATPd, only 295 pairs of corresponding amino acid residues were present in the Ca-Ca distance less than 3 Å, and their root mean square deviation was 2.0 Å. Several structural differences were observed between ArATPc and ArATPd as shown in Fig. 4. The 5th, 11th, and 13th α-helices of ArATPd are shorter than several amino acid residues in ArATPc, corresponding to H4, H8, and H10, respectively. Interestingly, the 9th α-helix (from Tyr141 to Val150) in the ArATPd molecule is transformed into a long loop (from Tyr202 to Phe222) between S5 and S6 of ArATPc. A loop (Val234–Ser246) between H11 and S8 of the small domain of ArATPc covers one end of the cleft formed at the interface between the large and small domains, although the corresponding loop is shorter in the ArATPd molecule and is unable to form a lid on the cleft.

As shown in Figs. 5 and 6, PLP is positioned at the bottom of the active site and forms an internal aldime bond (Schiff base linkage) with the catalytic residue Lys233 (corresponding to K258 in cAspATp). The phosphate moiety of PLP forms hydroxyster bonds with Oγ of Tyr259 (Tyr270 in cAspATp), N of Ala286 (Thr109 in cAspATp), Oγ of Ser232 (Ala257 in cAspATp), and Nε1 and Nε2 of Arg241 (Arg266 in cAspATp; Fig. 5A). The pyridine ring of PLP interacts with both methyl groups of Val201 (Ala224 in cAspATp) on one side, and on the other side with the phenyl ring of Phe121 (Trp140 in cAspATp), by stacking interaction. The 0–3 atom on the pyridine ring of PLP forms direct hydrogen bonds with the side chains of Asn171 (Asn194 in cAspATp) and Tyr202 (Tyr225 in cAspATp). The N1 atom on the pyridine ring forms a hydrogen bond with the side chain of Asp199 (Asp222 in cAspATp). Thus, PLP is fixed tightly at the bottom of the active center.

Gross Conformation and pH Stability—The gross conformation and pH stability of ArATPc were studied using CD spectroscopy at 25 °C. The CD spectrum in the region between 200 and 250 nm exhibited double negative minima at 209 and 223, which are characteristic of an α-helical structure (data not shown). The α-helical content is estimated to be approximately 40%, according to the method of Chen et al. (51). The enzyme is stable between pH 4 and 11 for 24 h at 25 °C.

Heat Stability—The residual ArATPc activity remaining after heating was measured to determine the half-life of the enzyme at 95 and 110 °C. The half-life of ArATPc is 30 min at 110 °C, and the enzyme is stable at 95 °C in 20 mM phosphate buffer (pH 6.5).
The heat capacity change of ArATPh was measured using differential scanning calorimetry from 0 to 125 °C at pH 6.5. The heat capacity change was only observed during the first differential scanning calorimetry from 0 to 125 °C at pH 6.5.

On the basis of the ArATPh structures, the surface area for one amino acid residue was calculated by dividing the accessible surface area of the dimer by total residue numbers to evaluate molecular compactness. The values of ArATPh and ArATPd are 28.0 and 33.6 Å², respectively. The lower surface area for one residue of ArATPh molecule might be due to tight packing of the polypeptide chain into the homodimer structure. Another prominent difference in ArATPh is the large number of charged residues (Asp, Glu, Lys, and Arg) on its molecular surface compared with the ArATPd. The occupancy of the charged residues in the accessible surface area of ArATPh and ArATPd molecules are 73.3 and 48.1%, respectively. On the contrary, the frequency of polar contacts less than 3.3 Å, including hydrogen bond and ion pair among these charged residues on the surface is decreased to 36.0% for ArATPh in comparison with the value, 51.3%, of ArATPd. The accessible surface of ArATPh has higher hydrophilicity with a lower number of ion pairs than that of ArATPd. The compact packing and the remarkably water-attractive surface of ArATPh are probably major factors contributing to its hyperthermostability.

The PLP molecule of ArATPh is fixed tightly with nine hydrogen bonds at the bottom of the active site cleft (Fig. 5A). One side of the pyridine ring of PLP interacts with the geminal dimethyl groups of Val201 (Ala224 in cAspATp), whereas the other side is stacked parallel with the phenyl ring of Phe121 (Trp140 in cAspATp). In AspATEc, the methyl group of Ala224 interacts with the pyridine ring of PLP on one side, and on the other side, the pyridine ring stacks to Trp140 with a 20° inclination angle. In the thermophilic enzymes of subfamily Iα, valine or isoleucine is found at the position corresponding to Val201 of ArATPh, whereas the residue is replaced with Ala in the mesophilic enzymes of subfamily Iα (2, 3). The interaction of Ala with PLP should be weaker than those of V/I in thermophilic aminotransferases because of the lack of a geminal di-methyl-CH/π interaction (53). In subfamily Iα of the thermophilic archaea (Fig. 1), the phenyl ring of Phe or Tyr, corresponding to Phe121 in ArATPh, always stacks to the pyridine ring of PLP. In subfamily Iα from the mesophilic organisms and subfamily Iγ from the thermophilic prokaryotes, these residues are replaced by tryptophan, which has a bulkier side chain with a wider surface area than does the phenyl ring (2). Consequently, a combination of the Phe121 and Val201 residues stacking tightly to the pyridine ring of PLP may contribute to the hyperthermophilic properties of ArATPh. Further crystallographic studies are in progress to better understand the mechanisms underlying the hyperthermostability of this enzyme.

PLP-binding Structure of ArATPh—In the PLP molecule of ArATPh, the number of hydrogen bonds fixing the phosphate...
The phosphate moiety is reduced from six to five (Fig. 5A), because of replacement of the Ser_{255} residue, which is conserved in both AspAT_Ec and ArAT_Pd (6, 7). The large conformational change of the phosphate moiety of PLP is induced by a shift in the side chains of Ala_{96}, Ser_{232}, and Arg_{241} from the corresponding residues in AspAT_Ec. The phosphate moiety moves parallel to the plane of the pyridine ring of PLP, whereas the pyridine ring is conserved at the same position as in AspAT_Ec. The movement of the phosphate moiety in the opposite direction might be a positive adjustment of the cofactor to compensate for changes in the secondary structure that account for its hyperthermostability. Interestingly, the O_η position of Tyr_{202} in ArAT_Ph is almost identical to that of the corresponding Tyr_{225} residue of AspAT_Ec (Fig. 5A), whereas the coordinates of the main chain parts in both Tyr residues are shifted by more than 2 Å. The Tyr residues of both ArAT_Ph and AspAT_Ec are close enough to form a hydrogen bond with O_3H of PLP. The position of the ω-carboxyl of Asp_{199} forming a hydrogen bond with N1H of PLP is also identical to that of Asp_{222} of AspAT_Ec. These results strongly indicate that the pyridine ring must be fixed precisely at the conserved position in the active center of ArATs to make the cofactor fully active, although the phosphate moiety can be positioned according to the steric requirements. The O_η of the Tyr_{202} residue of ArAT_Ph can also form a hydrogen bond with the imino group of the Schiff base. The angle of the imino proton on the C=N plane of ArAT_Ph is sufficient to form a hydrogen bond with Tyr_{202}, however, the corresponding angle in AspAT_Ec seems less suitable to form a hydrogen bond with Tyr_{225} (Fig. 5A). The pK_a of the Schiff base of ArAT_Ph was determined to be 5.1, which is the lowest value ever reported;
Fig. 4. Superimposition of ArATPh (green) on ArATPd (red) (7). The figure was produced using the program Turbo-Frodo. The PLP molecule of ArATPh is represented by a space-filling model.

Fig. 5. The PLP binding structure of ArATPh. A, the stereoview for the superposition of ArATPh (red) and AspATEc (blue) by the PLP fitting. Although the overall structure of AspATEc including the PLP binding profile (6) is quite similar to that of ArATPd (7), the AspATEc was selected as a reference structure for the superposition because of its general popularity historically. The residue numbers indicate the positions in the ArATPh molecule (red). Dotted lines indicate the hydrogen bonds in ArATPh (red). B, nomenclature of atoms for PLP.
Fig. 6. The substrate-binding model for ArATPh represented by stereoview. The front view of the active site with Tyr. The model structure is presented in a sphere with a 15-Å radius surrounding the substrate. The subunits A and B are colored green and pink, respectively. The substrates and PLP are colored blue and red, respectively. The possible hydrogen bonds are represented as dashed lines. The yellow figure, 3.4, indicates the distance (Å) between the internal aldimine bond and the α-amino group of Tyr.

The pKa values of AspATc, T. thermophilus AspAT, and ArATc were reported to be 6.8 (54), 6.1 (8), and 6.65 (55), respectively. This low pKa value is probably due to rotation of the C=N plane of the Schiff base against the pyridine ring of PLP to control hydrogen bonding between the imino group and the Tyr^352 residue and may also be due to the unique environment around the PLP molecule caused by changes in the residues stacked to PLP (Fig. 5A).

Active Site Structure and Substrate Binding Models—The active site structure with the best substrate, Tyr, is shown in Fig. 6. The α-carboxylate of Tyr was fixed at the active site by two salt bridges with Arg^362 (corresponding to R386 in cAspATp) and three hydrogen bonds with Gln^34, Asn^171, and Tyr^230. The phenyl ring of Tyr and the aromatic group of Phe^121 undergo an energetically favorable “edge-to-face” interaction (56), and the aromatic ring of Tyr^356 is located very closely, but not in parallel, to the phenyl ring of Tyr. Thus, the best substrate can be trapped in the hydrophobic pocket formed by Phe^121, Tyr^356, the pyridine ring of PLP, Met^260 (corresponding to Arg^292 in cAspATp), and Val^122 (Glu^141 in cAspATp). In this binding model, the OH group of Tyr is located at a distance sufficient to form hydrogen bonds with Oy1 of Thr^264e (Ser^296e in cAspATp) and with the phosphate moiety of PLP. The internal aldimine bond between PLP and Lys^313 (corresponding to Lys^258 in cAspATp) is located so close that a new external aldimine bond can be formed between PLP and the α-amino group of Tyr.

Another binding model was formed with Glu, one of the best acidic substrates. A water molecule (Xaa^126a) is present at the center of three adjacent groups: the α-carboxyl of Glu, the Oy1H of Thr^264e, and the phosphate residue of PLP. The proximity (within 3 Å) of the water molecule and the adjacent residues allows formation of a hydrogen-bond network among them. The water molecule (Xaa^126a) may be important in binding Glu to the active center, as indicated by the reportedly complex structure of ArATPh with maleate (7). Furthermore, the γ-carboxyl group of Glu is parallel to the phenyl ring of Tyr^59g, suggesting a van der Waals’ interaction between the two groups. This sort of weak interaction may be important for the recognition of C5 substrates (Glu and 2OG) in amino acid aminotransferases, because the Y70S mutant of AspATc is reportedly less active against these substrates (57). The phenyl ring at position 70 is essential for the recognition of the Glu-2OG pair as substrates. Hence, in the binding model of ArATPh, both ends of the acidic substrate, Glu, are fixed at the active center of the enzyme by three major interactions: 1) salt bridges between Arg^362 and the α-carboxylate of Glu; 2) two hydrogen bonds located between Gly^34 and the α-carboxylate, and between Gly^34 and α-amino groups of the substrate, and 3) the capturing of the γ-carboxylate by the hydrogen bond network through the water molecule Xaa^126a and by a weak interaction with Tyr^59g. The low activity against Asp is explained in two ways: 1) the lack of an arginine residue corresponding to Arg^292 of cAspATp, which interacts with the distal carboxylate of the acidic substrate and 2) the lack of a hydrogen bond network through the water molecule Xaa^126a and no interaction with Tyr^59g, because of a lack of one methylene unit at the γ position.

Substrate Specificity of ArATPh—As shown in Tables I and II, ArATPh prefers the substrates in the following order in $k_{\text{cat}}/K_i$: Tyr > Phe > Glu > Trp > His > Met > Leu > Asp > Asn. The substrate specificity differs from those of the mesophilic ArATs, including ArATPh, with the preference in $k_{\text{cat}}/K_i$ being Tyr > Phe = Asp > Trp > Glu (41). Thermostable ArATs from Pyrococcus furiosus and Methanococcus aeolicus were also reported to have distinct substrate specificity in $k_{\text{cat}}/K_i$: Phe > Trp > Tyr (52, 58). Consequently, ArATPh has a novel substrate specificity compared with other ArATs.

Aminotransferases are increasingly applied to the large scale synthesis of unnatural and nonproteinogenic amino acids (1). Typically exhibiting relaxed substrate specificity, rapid reaction rates, and no need for cofactor regeneration, they possess many characteristics that make them useful for biocatalysis. Because of its novel substrate specificity and high level of resistance to organic solvents (data not shown), ArATPh will continue to be a useful biocatalysis for the synthesis of unnatural compounds.

Acknowledgments—We thank Miyuki Ishimura for assistance with the differential scanning calorimetry analysis and valuable discussions. Koichi Honda is gratefully acknowledged for his practical advice and valuable discussions.
