Antibody 15A9, raised with 5'-phosphopyridoxyl (PPL)-N-acetyl-L-lysine as hapten, catalyzes the reversible transamination of hydrophobic D-amino acids with pyridoxal 5'-phosphate (PLP). The crystal structures of the complexes of Fab 15A9 with PPL-L-alanine, PPL-D-alanine, and the hapten were determined at 2.3, 2.3, and 2.5 Å resolution, respectively, and served for modeling the complexes with the corresponding planar imine adducts. The conformation of the PLP-amino acid adduct and its interactions with 15A9 are similar to those occurring in PLP-dependent enzymes, except that the amino acid substrate is only weakly bound, and, due to the immunization and selection strategy, the lysine residue that covalently binds PLP in these enzymes is missing. However, the N-acetyl-L-lysine moiety of the hapten appears to have selected for aromatic residues in hypervariable loop H3 (Trp-H100e and Tyr-H100b), which, together with Lys-H96, create an anion-binding environment in the active site. The structural situation and mutagenesis experiments indicate that two catalytic residues facilitate the transamination reaction of the PLP-D-alanine aldimine. The space vacated by the absent L-lysine side chain of the hapten can be filled, in both PLP-alanine aldimine complexes, by mobile Tyr-H100b. This group can stabilize a hydroxide ion, which, however, abstracts the Cα proton only from D-alanine. Together with the absence of any residue capable of deprotonating Cα of L-alanine, Tyr-H100b thus underlies the enantiomeric selectivity of 15A9. The reprototation of Cα of PLP, the rate-limiting step of 15A9-catalyzed transamination, is most likely performed by a water molecule that, assisted by Lys-H96, produces a hydroxide ion stabilized by the anion-binding environment.

The most often used approach for generating catalytic antibodies is to elicit antibodies against a haptenic group that mimics the transition state of the target reaction (1). The crystal structures of several antibodies obtained in this way validate this procedure and show the catalytic antibodies to bind and stabilize the transition state through van der Waals, hydrogen bonding, and electrostatic interactions (2, 3). Only by chance, these antibodies possess residues at the combining site that directly participate in the covalence changes of the reaction.

The lack of catalytic residues is one of the reasons why the rate enhancement brought about by catalytic antibodies only occasionally approaches that of enzymes. The “bait and switch” strategy, which uses a charged hapten to elicit a programmed chemically reactive charged residue (4, 5), and the related procedure of “reactive immunization” (6) have been developed for obtaining catalytic antibodies endowed with reactive residues. Another possibility is to expand the catalytic scope of antibodies by incorporating a nonproteinaceous cofactor (7) that contributes to the catalytic efficacy while the antibody ensures substrate specificity and possibly reaction specificity. As yet, the structure of only one catalytic antibody that operates in concert with a cofactor has been elucidated (8).

Pyridoxal 5'-phosphate (PLP) is probably the most versatile organic cofactor of enzymes; in amino acid metabolism, PLP-dependent enzymes synthesize, degrade, or interconvert amino acids. We have previously described the production of antibodies with PLP-dependent catalytic activity (9–12). Monoclonal antibody 15A9 was generated against N5'-phosphopyridoxyl(PPL)-L-lysine conjugated to maleylated keyhole limpet hemocyanin 1a (Fig. 1A), hapten 1b being a stable analog of the covalent PLP-L-amino acid intermediates 4 and 5 (Fig. 1B). Antibody 15A9 was selected among the hapten-binding antibodies because its combining site also accommodated the planar PLP-amino acid aldimine 4 (9, 10). Although the hapten contains the L-enantiomer of lysine, antibody 15A9 catalyzes the transamination reaction of PLP exclusively with hydrophobic D-amino acids (Fig. 1B), D-alanine being the best substrate, with $k_{cat} = 0.42 \text{ min}^{-1}$ at 25 °C. In comparison with the unassisted transamination reaction of the PLP-amino acid aldimine 4, the rate-enhancing effect of the antibody is $5 \times 10^5$ (9). Here we report the high resolution crystal structures of Fab 15A9 in complex with diverse PLP-amino acids. On this basis and in light of amino acid substitution experiments, we propose a catalytic mechanism for this antibody and discuss the origin of its unexpected enantiomeric selectivity. The striking mechanistic analogies between 15A9 and PLP-dependent enzymes are consistent with the chemical necessities of protein-assisted pyridoxal catalysis as previously deduced from structural comparisons of PLP-dependent enzymes.

The abbreviations used are: PLP, pyridoxal 5'-phosphate; mAspAT, chicken mitochondrial aspartate aminotransferase; PPL, N5'-phosphopyridoxyl; bis-Tris-propane, 1,3-bis[tris(hydroxymethyl)methylamino] propane; DaAT, D-amino acid aminotransferase from Bacillus species YM-1.
EXPERIMENTAL PROCEDURES

Preparation and Structure Determination of the Protein-Ligand Complexes—The proteolytic Fab fragment of the monoclonal antibody 15A9 was produced and purified as described (14). Recombinant wild-type Fab 15A9 was produced and purified as described previously (15). The PPL-amino acid derivatives were obtained by reduction of the spontaneously forming PLP-aldimine adducts 4 (see Fig. 1) with sodium borohydride (9). The three complexes with PPL-amino acids crystallized in 0.2 M sodium iodide, 50 mM sodium acetate, pH 6.0, 30% polyethylene glycol 3350 at equimolar concentrations of protein and ligands. Data were recorded on beamlines ID14-2 and ID14-3 at the European Synchrotron Radiation Facility in Grenoble and were processed with DENZO and SCALEPACK (16) (Table 1). The structure of the hapten-liganded Fab 15A9 (IgG1, H9260) was solved by molecular replacement with AMoRe (18), using the Fv domain and the CL-CH1 dimer of Fab A5B7 (Ref. 19) (Protein Data Bank code 1clo) as models and in turn served as a model for solving the structures of the PPL-alanine complexes. The atomic models were refined by alternating cycles of model rebuilding with the program O (20) and of refinement with CNS (21).3

Preparation of Fab 15A9 Variants—The mutants were prepared by polymerase chain reaction from plasmid pASK85-15A9 (15) using the QuickChange site-directed mutagenesis kit from Stratagene and the following primer pairs: YH100bFa, 5'-GGCTCTGTATGTAACCTCCAGGCTTTTCG-3'; YH100bFb, 5'-GCAACCAGGCTCTGAAGTTACCATACGAGCC-3'; YH100bKa, 5'-GGCTCTGTATGTAACCTGTTACAGGTTACCATACGAGCC-3'; YH100bKb, 5'-GCAACCAGGCTCTGTTACCATACGAGCC-3'; KH96Aa, 5'-CCACTTTATTTACGTGTAAGAGATGCGGGCTCTGTTACCATACGAGCC-3'; KH96Ab, 5'-CCACTTTATTTACGTGTAAGAGATGCGGGCTCTGTTACCATACGAGCC-3'. The mutants were expressed and purified as described previously (15).

Measurement of Catalytic Activities—All assays were performed in 50 mM bis-Tris-propane, 140 mM NaCl, pH 7.5, at 25 °C. The transaminase activity of recombinant wild-type and mutant Fab 15A9 was measured by following the increase in absorbance at 325 nm due to the production of pyridoxamine 5'-phosphate. PLP (400 μM) and D-alanine (200 mM) were preincubated for 15 min at 25 °C before the addition of 5 μM Fab 15A9. For monitoring the β-elimination reaction of β-chloro-D-alanine, 1 mM PLP, 10 mM β-chloro-D-alanine, and 5–10 μM Fab 15A9 were incubated for 15 min at 25 °C. Pyruvate produced was then determined with lactate dehydrogenase and NADH.

Measurement of Dissociation Equilibrium Constants—The quenching of the intrinsic fluorescence of 0.25–0.40 μM Fab 15A9 (excitation at 280 nm, emission measured at 342 nm) was determined as a function of the concentration of PPL-D-alanine.

3 The coordinates of the complexes of 15Ag with PPL-L-alanine, PPL-D-alanine, and PPL-L-lysine have been deposited in the Protein Data Bank (PDB codes 1wc7, 2bmk, and 1wcb, respectively).
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RESULTS AND DISCUSSION

Structure of the Combining Site of Antibody 15A9—Antibody 15A9, which was elicited with PPL-N*-acetyl-L-lysine 1b (Fig. 1A), selectively binds the adduct of PLP with l-norleucine in clear preference to that with d-norleucine (9). With shorter amino acids, the enantiomeric binding selectivity is reduced, so that the hapten occupies a shallow cleft (Fig. 2A). A similar situation is observed in other catalytic antibodies possessing a bulky residue at the position preceding H101 (2), the corresponding bulky residue in 15A9 being Phe-H100f (11). The homology model that has recently been proposed for 15A9 (15) is substantially different from the crystal structure.

The high affinity of IgG 15A9 for its hapten (Kd = 26 nM) compared with that for PPL-l-alanine or PPL-d-alanine (1.4 and 1.5 μM, respectively) is attributed to stacking interactions of the N*-acetyl-L-lysine moiety of the hapten with Tyr-L32, Tyr-H100b, and Trp-H100e (Fig. 2, B and C). These interactions are consistent with the order of preference of amino acids in aldimine binding, which is as follows: N*-acetyl-lysine > norleucine > alanine > glycine (24). Tyr-H100b and Trp-H100e belong to the hypervariable loop H3, which is most diverse both in length and sequence among the hypervariable loops shaping the antibody-combining sites. The significant stabilization of the antibody-hapten complex achieved by stacking interactions with the haptenic N*-acetyl-L-lysine moiety might thus have favored the selection of tyrosine at position H100b and tryptophan at position H100e. Indeed, an important difference between the three structures comes from the absence of the lysine side chain of the hapten in the PPL-l-Lys and PPL-d-Ala structures. Tyr-H100b, which adopts the same conformation in the two molecules of the asymmetric unit of the PPL-N*-acetyl-L-Lys complex, shows flexibility in the PPL-d-alanine structures, assuming different conformations in the two molecules of the asymmetric unit of the PPL-d-alanine structures (Fig. 2D) and having high B-factors in the PPL-l-alanine structure.

Two iodide ions from the crystallization solution, I1 and I2, were identified with full occupancy in the combining site of the 15A9-hapten complex; I2 fixes the conformation of loop H3, whereas I1 engages in an ion pair with the e-amino group of Lys-H96 and stacks against the lysine side chain of the hapten and the indole group of Trp-H100e (Fig. 2C), thereby locating an anion-binding site in the active site of 15A9. Sodium iodide at the concentration used in the crystallization medium (0.2 M) decreased the transaminase activity of 15A9 in solution only by 42%.

Comparison of the Active Sites of Antibody 15A9 and PLP-dependent Aminotransferases—The interactions of 15A9 with the reduced adduct PPL-α-alanine (Fig. 3A) were compared with those occurring in the crystal structures of chicken mitochon-

TABLE 1

Data collection and refinement statistics for Fab 15A9 complexed with hapten 1b, PPL-d-alanine 2 and PPL-l-alanine 3

| Data collection | Hapten 1b | PPL-d-Ala 2 | PPL-l-Ala 3* |
|-----------------|-----------|-------------|-------------|
| Space group     | P1        | P2          | P2          |
| No. of molecules in the asymmetric unit | 2         | 2           | 2           |
| Cell dimensions |           |             |             |
| α, β, γ (Å)     | 57.9, 60.3, 64.6 | 63.1, 81.2, 79.3 | 63.5, 81.7, 79.3 |
| a, b, c (Å)     | 79.4, 78.2, 80.8 | 90, 90.3, 90 | 90, 90, 90 |
| Resolution (Å)  | 25.2-25 (2.54-2.5) | 40-2.3 (2.34-2.3) | 40-2.3 (2.34-2.3) |
| No. of observations | 54,373 | 120,139 | 113,697 |
| Unique reflections | 28,322 | 32,263 | 34,388 |
| Rmerge (%)      | 0.076 (0.195) | 0.091 (0.592) | 0.125 (0.696) |
| I/σ(I)          | 15.9 (5.1) | 14.2 (2.5) | 9.3 (2.0) |
| Completeness (%)| 95.8 (94.3) | 99.9 (99.9) | 99.8 (99.9) |
| Redundancy      | 1.9 (1.8) | 3.7 (3.6) | 3.31 (3.09) |
| Refinement      |           |             |             |
| Resolution (Å)  | 40-2.5 | 40-2.3 | 20-2.3 |
| Rmerge/Rwork (%)| 0.223/0.290 | 0.210/0.272 | 0.223/0.309 |
| No. of residues |           |             |             |
| Protein         | 872 | 870 | 869 |
| Ligand/iodide ions | 2/9 | 2/6 | 1/5 |
| Water           | 473 | 354 | 120 |
| B-factors (Å²)  |           |             |             |
| Protein         | 21.6 | 30.6 | 48.1 |
| Ligand/iodide ions | 15.2/58.8 | 27.4/58.7 | 41.5/77.4 |
| Water           | 19.3 | 32.2 | 40.2 |
| Root mean square deviations |           |             |             |
| Bond lengths (Å) | 1.44 | 1.38 | 1.58 |

* The crystal of the 15A9-l-Ala complex was pseudomerohedrally twinned (monoclinic space group with β = 90°) with a twinning fraction of 0.46 and a twin operator (h, k, -l) (14). The structure was refined with SHELXL (17).
* Of the data, 10% were set aside for the Rwork calculation during the entire refinement for the PPL-l-Lys and PPL-d-Ala complex and 5% for the PPL-l-Ala complex.
drial l-aspartate aminotransferase (mAspAT) in complex with the PLP-l-aspartate ketimine intermediate (25) (Fig. 3B) and d-amino acid aminotransferase from Bacillus species YM-1 (DaAT) in complex with PPL-d-alanine (26) (Fig. 3C). These two enzymes do not show any mutual sequence similarity and have different folds and different enantiomeric selectivities. mAspAT is one of the most thoroughly characterized aminotransferases; in particular, the catalytic activity of its K258H variant lacking the PLP binding lysine residue has been explained on the basis of its crystal structure (27). DaAT has the same enantiomeric selectivity as 15A9.

The PLP moiety is bound in a quite similar way in all three cases (Fig. 3). First, in 15A9, two ionic interactions of Asp-H95 and Arg-L91 with the nitrogen and the phenolic oxygen atom of PLP, respectively, indicate that the hydroxyypridined moiety is bound in its zwitterionic form. In both mAspAT and DaAT, the pyridine nitrogen atom is also protonated as it makes an ionic interaction with an aspartate residue, and the phenolic oxygen atom of PLP is negatively charged. Second, the pyridine ring of the cofactor is engaged in stacking interactions, in 15A9, through an edge-to-face aromatic interaction with Phe-L96 as well as in a cation–π interaction with Lys-H96. The ionic interactions maintaining the pyridine nitrogen atom in its protonated form and the aromatic interactions contributing to the extended pyridine-aldimine resonance system create an electron-withdrawing effect that weakens the Co–H bond. Third, in both the antibody and the enzymes, the nonbridging oxygen atoms of the phosphate group of PLP participate in ionic interactions with an arginine residue and make hydrogen bonding interactions. Finally, the conformation of the planar 15A9-bound coenzyme-substrate adduct (see below) appears to be similar to that usually observed in PLP-dependent enzymes, since the dihedral angle (C4′–N Ca C4 COO−) of the PPL-d-alanine adduct is close to 180°. This cisoid conformation with the imine nitrogen on the same side of the C4′–C4′ bond as O3′ (Fig. 1) (i.e. with maximum distance between the phosphate and the α-carboxylate group) is also observed in mAspAT and DaAT; it corresponds to the minimum energy conformation of the adduct and is assumed to result in stronger binding to the protein (28, 29).

An important difference between 15A9 and both enzymes is the much weaker interaction with the amino acid moiety of the aldime adduct in the antibody; only one α-carboxylate oxygen atom directly interacts with 15A9 through a hydrogen bond with His-L34, whereas in both enzymes the α-carboxylate group engages in a bidentate ionic interaction with an arginine residue. In 15A9 and DaAT, unlike mAspAT, the loose interactions with the methyl side chain of alanine are consistent with their not strictly defined substrate specificity; in 15A9, hydrophobic d-amino acids are generally accepted as substrates.

In all aminotransferases, the active-site lysine residue that covalently binds the cofactor is also responsible for the proton abstraction from Coa of the amino acid and the reprotonation at C4′ of PLP in the tautomerization of aldime 4 to ketimine intermediate 5 (29, 30). Substitution of Schiff base-forming Lys-258 in mAspAT with alanine, arginine, or cysteine leads to inactivation. However, when Lys-258 is changed to histidine, a residual activity of 0.1% of that of the wild-type enzyme (kcat = 4.2 min−1 at 25 °C) persists (31). The K258H mutant of mAspAT has a catalytic activity only 1 order of magnitude higher than that of antibody 15A9 and catalyzes the same reaction steps: formation of the PLP-amino acid aldime 4, tautomerization to the ketimine 5, and hydrolysis to give the oxo acid product 6 and pyridoxamine 5′-phosphate (Fig. 1B). The crystal structure of mAspAT K258H shows that the imidazole group of His-258 is too far away from Coa of the amino acid in the aldime adduct for acting directly as proton donor/acceptor. The prototropic shift was therefore suggested to proceed through His-258 and an intervening water molecule that, however, was not observed in the crystal structure (32). Like mAspAT K258H, antibody 15A9 does not bind covalently the aldehyde group of PLP, since this feature was not included in the immunization and selection strategies. Noncovalent binding of PLP is indicated by the similar Kcat’ values for PLP and PMP and by resistance of the catalytic activity to the reducing agent borohydride (9). In addition, the present structural study shows that only one lysine residue, Lys-H96, is found in the active site of 15A9, and its ε-amino group can neither form an imine bond with PLP nor deprotonate Coa. Therefore, the catalytic activity and the enantiomeric selectivity of 15A9 for d-amino acids residues are not due to the correct positioning of a catalytic lysine residue as in the case of mAspAT and DaAT. The opposite enantiomeric selectivities of these enzymes originate from the location of the catalytic lysine residue on opposite faces.

FIGURE 2. Structure of the combining site of Fab 15A9 in complex with hapten 1b and PPL-d-alanine 3. A, the molecular surface of the combining site of the 15A9hapten 1b complex. The molecular surface shown in transparent pink for the light chain and light yellow for the heavy chain was drawn with the program GRASP. Residues Ser-H35, Trp-H47, Trp-H103, and the hypervariable H3 loop (shown as a worm) are indicated in red, and the hapten is shown in yellow. The bulky residue Phe-H100f, shown in cyan, is at the bottom of the cavity, which makes the binding site shallow. B, stereo view of the active site of the 15A9hapten 1b complex. The ligand is in tan, and iodide ions and water molecules that are conserved in both subunits are indicated as green and red crosses, respectively. The Co atoms of residues L26–L34, L50–L52, L91–L96, H26–H33, H52–H56, and H95–H103 in hypervariable loops L1, L2, L3, H1, H2, and H3 are shown in dark blue, light blue, green, yellow, magenta, and orange, respectively. The residues that directly interact with the hapten are represented: Tyr-L32, His-L34, Arg-L91, Tyr-L94, Phe-L96, Tyr-H33, Arg-H52, Asp-H95, Lys-H96, Tyr-H100b, Trp-H100e; the amino acid sequence of 15A9 and the numbering of the residues are to be found in Ref. 10. The acetyl part of hapten 1b, which was not seen in the electronic density, is expected to extend the stacking interactions with Tyr-H100b. The (CS C4 C4′ Nua) dihedral angle deviates from planarity by 40°, and the (CS C4 C4′ Nua) angle deviates by 7° in both molecules 1 and 2 of the asymmetric unit. The phosphate group of the represented molecule 2 interacts with Ser-H161# (2.9 Å) and Lys-L169# (3.5 Å) of molecule 1 (not represented). The hapten buries 80% of its accessible surface inside the combining site of the antibody. The amino acid part of the hapten and the phosphate group of the coenzyme are largely solvent-accessible, whereas the pyridine moiety is completely buried. C, a Fε – Fε electron density map, calculated by omitting the hapten and the iodide ions and a 3.5 Å spherical region around them is contoured at the level of 2.0 σ and is superimposed on the active-site structure. D, superposition of the structures of the active sites of the two molecules in the asymmetric unit of 15A9 in complex with PPL-d-alanine 3. The structure of molecule 1 (cyan) is superimposed onto that of molecule 2 (yellow) (root mean squared deviation of 0.19 Å). A Fε Fε electron density map omitting the PPL-d-alanine ligands and a 3.5 Å spherical region around them is contoured at the level of 2.0 σ and superimposed onto the active-site structures.
FIGURE 3. Comparison of the active sites of Fab 15A9, mAspAT, and DaAT. A, Fab 15A9 in complex with PPL-D-alanine. Hydrogen bonds are indicated as red dashed lines, and the water molecules or iodide ions that are conserved in the two molecules of the asymmetric unit are indicated as red and green crosses, respectively. The (C5 C4' Nα) dihedral angle of the ligand deviates from planarity by 42 and 45° and the (C4 C4'/H11032 N/H9251) dihedral angle by 42 and 36°, in molecules 1 and 2, respectively. One iodide ion fixes the conformation of hypervariable loop H3 in both molecules, and the four other iodide ions are involved in crystallographic contacts. The iodide ion interacting with the lysine side chain of the hapten in the hapten-15A9 complex (Fig. 2) is replaced in the 15A9/PPL-D-alanine complex by a water molecule W positioned 4.6 Å (4.9 Å) from C/H9251, 3.6 Å (3.8 Å) from C4'/H11032, and 3.2 Å (2.7 Å) from Lys-H96, in molecules 1 and 2, respectively. The pyridine ring of the cofactor is engaged in a stacking interaction with Phe-L96 and Lys-H96, and the positively charged pyridine nitrogen atom is stabilized by an ion pair with the carboxylate group of Asp-H95. The phenolic oxygen atom makes an ion pair/hydrogen bond with Arg-L91. One carboxylate oxygen atom of the amino acid substrate is bound to His-L34, and the other one interacts with water molecules. The phosphate group forms an ion pair with Arg-H52 and is hydrogen-bonded to the phenolic oxygen atoms of Tyr-L94 and Tyr-H33. Substitution of Arg-L91, Tyr-L94, or Tyr-H33 with alanine indeed leads to a marked decrease in both the affinity for PPL-amino acids and the catalytic activity (15). The 2-methyl group of PLP is in contact with Phe-L96, Trp-H100e, Asp-H95, and Arg-L91. Lys-H96 is the only lysine residue found in the active site of 15A9, with its ε-amino group positioned 7.3 Å (6.8 Å) away from C/H9251 and 5.6 Å (4.9 Å) away from C4'/H11032 of PPL-D-alanine, in molecules 1 and 2, respectively.

B, mAspAT in complex with ketimine PLP-L-aspartate (Protein Data Bank code 1MAP). The pyridine ring of the cofactor is engaged in a face-to-face aromatic interaction with Trp-140. The positively charged pyridine nitrogen atom is stabilized by an ion pair with the carboxylate group of Asp-222, and the negatively charged phenolic oxygen is hydrogen-bonded to the hydroxyl group of Tyr-225. The ε-carboxylate group is bound through a bidentate salt bridge/hydrogen bond with Arg-386. The phosphate group forms an ion pair with Arg-266 and is hydrogen-bonded to the phenolic oxygen atoms of Thr-205 and Tyr-70# from the adjacent subunit. The ε-amino group of the catalytic base Lys-258 is equidistant from atoms C4'/H11032 and C/H9251 (3.4 and 3.6 Å, respectively) and is located on the si face of the coenzyme adduct. C, DaAT in complex with PPL-D-alanine (Protein Data Bank code 3DAA). The pyridine ring of the cofactor is engaged in stacking interactions with Leu-201 and the backbone of Ser-180. The positively charged pyridine nitrogen atom is stabilized by an ion pair with the carboxylate group of Glu-177, and the negatively charged phenolic oxygen makes hydrogen bond interactions through a bridging water molecule with the Nζ2 atom of His-100# from the second subunit and the carbonyl group of Ser-179. The carbonyl group of Ser-179, the carboxylate group of Gln-104, and the carbonyl group of Ser-179. The carboxylate forms a bidentate salt bridge with Arg-98#, and one of its oxygen atoms also interacts with the hydroxyl group of Tyr-31. The side chain of PPL-D-alanine points into a large pocket so that a wide variety of d-amino acid substrates can be accommodated. The phosphate group makes an ion pair with Arg-50 and is hydrogen-bonded to the hydroxyl groups and main chain nitrogen atoms of Thr-205 and Thr-241 and the main chain nitrogen atom of Ile-204. The ε-amino group of the catalytic base Lys-145 is equidistant from atoms C4' and Cα (3.7 and 3.4 Å, respectively) and is located on the re face of the coenzyme adduct.
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of the coenzyme (26); PLP is bound to DaAT with its re side facing the protein and the catalytic lysine residue, whereas in mAspAT, the si side faces the protein (Fig. 3).

Docking of the Aldimine Intermediates into the Active Site of 15A9—The origin of the catalytic activity of the antibody and of its enantiomeric selectivity for d-amino acid substrates is best discussed by modeling the planar aldimine intermediate 4 into the active site of 15A9. The PLP-d-alanine and PLP-L-alanine aldimine intermediates 4 of transamination (Fig. 1B) were manually docked into the combining site of Fab 15A9 based on the PPL-d-Ala and PPL-l-Ala structures (Fig. 4). In modeling, two essential features of the aldimine adduct were considered. First, in contrast to the reduced PPL-alanine adduct, the aldimine reaction, reprotonation at C4’ seems to be the rate-limiting step in transamination (10).

The lower efficiency of the catalytic antibody compared with that of PLP-dependent enzymes appears to be due to the absence of a properly positioned catalytic lysine residue in the active site of 15A9 (Fig. 3A). Instead, Tyr-H100b appears to account for the enantiomeric selectivity of 15A9 toward d-amino acids. In the complexes with PLP-alanine aldimine, mobile Tyr-H100b can be oriented without steric hindrance in the space vacated by the absence of the l-lysine side chain of the hapten. Its hydroxyl group is thereby suitably oriented to stabilize by hydrogen bonding a hydroxide ion that acts as the Ca-deprotonating base. In this orientation, Tyr-H100b as well
as the hydroxide ion carrying out the deprotonation are located on the same face of the cofactor and in a similar position as the catalytic base Lys-145 in DaAT (Fig. 3C), which is specific for D-amino acids. Therefore, the positions of the catalytic residue Tyr-H100b and the hydroxide ion explain why the antibody catalyzes proton abstraction from Coa of D-amino acids. Alternatively, the nearby anion-binding environment of Lys-H96 and Trp-H100e might stabilize the hydroxide ion and be catalytically effective without support by Tyr-H100b. The notion of an essential role for Tyr-H100b in 15A9 in catalysis is, however, supported by mutagenesis experiments; the substitution of Tyr-H100b with alanine has previously been shown to lead to inactivation without reducing the affinity of Fab 15A9 for PLP, but without reducing the catalytic efficacy of the cofactor, determines along which of the numerous potential pathways the adduct will proceed. Antibody 15A9 is remarkable reaction- and enantiomer-specific, since no reactions other than transamination with D-amino acids and loss of activity of the YH100bA mutant is due to the absence of the hydroxyl group rather than to the absence of the hydrophobic side chain, Tyr-H100b was substituted with phenylalanine. The catalytic activity of the YH100bf mutant was determined in the transamination reaction of PLP with D-alanine and in the β-elimination reaction of β-chloro-d-alanine (Fig. 1C). Both activities are markedly reduced, whereas the affinity for PPL-D-alanine is essentially the same as that of wild-type Fab (Table 2). The conclusion that Tyr-H100b positions and stabilizes through a hydrogen bond a hydroxide ion that deprotonates Coa of the PLP-D-alanine aldime is supported by the higher activities of the mutant in which Tyr-H100b was replaced by a lysine rather than by a phenylalanine residue (Table 2). Apparently, the ε-amino group of lysine partially substitutes the aromatic hydroxyl group in positioning the hydroxide ion involved in deprotonation of Coa. The effect of the YH100bf substitution on the β-elimination activity (10-fold decrease) is higher than on the transaminase activity (3-fold decrease), very likely because in transamination the reprotonation at C4’ is rate-limiting.

In the model of the 15A9-PLP-L-alanine complex (Fig. 4B), the Ca hydrogen points toward a chemically inert region of the antibody, the backbone carbonyl groups of Arg-L91 and Ser-L92 being the nearest neighbors of Ca. Moreover, no water molecule suitably positioned to abstract the Ca hydrogen was observed in the PPL-L-alanine structure, nor could one be modeled into the PPL-L-alanine complex without significant rearrangement of the protein structure. Clearly, since no amino acid side chain or water molecule occupies the position corresponding to the catalytic Lys-258 in mAspAT (with L-enantiomeric specificity), relative to the cofactor (Fig. 3B), 15A9 cannot be active toward the L-enantiomer of alanine.

The reprotonation at C4’, similar to the deprotonation at Coa, is not carried out by a general acid/base group of 15A9. The model of the 15A9-PLP-d-alanine complex shows Lys-H96, which is the residue with the side chain nearest to C4’, to be located on the same side of the cofactor as Tyr-H100b but too far away to act directly at C4’ as a general acid (Fig. 4A). Nevertheless, in the 15A9-PPL-D-alanine structure, a water molecule W is hydrogen-bonded to the ε-amino group of Lys-H96 and positioned only 3.6 Å (3.8 Å) away from C4’ (Fig. 3B). Since W is located at the anion-binding site occupied by I1 in the hapten complex (Fig. 2B) and has a very low B-factor (18 or 8 Å²), we propose that it could protonate C4’ to form the ketimine intermediate 4 and thereby transform into a hydroxide ion. Lys-H96, which presumably is protonated at pH 7.5, is positioned at the correct angle and distance for stabilizing this hydroxide ion. The pH rate profile of the 15A9-catalyzed transamination reaction of PLP with D-alanine between pH 6 and 10 showed an optimum at pH 7.5, the rate measured at this pH value corresponding to kcat. The inflection point in the basic limb at pH 9 might be due to Lys-H96, and the inflection point in the acidic limb at pH 6.8 could reflect the deprotonation at Ca by a hydroxide ion, which becomes rate-limiting at acidic pH. In accord with Lys-H96 being important for the reprotonation at C4’, substitution of this residue with alanine reduces the transamination activity to 3% of that of wild-type Fab, whereas the effect on the β-elimination activity, which does not require reprotonation at C4’ (10), is considerably smaller (50% residual activity; Table 2).

Conclusions—Similar to PLP-dependent enzymes, antibody 15A9 modulates the intrinsic chemical disposition of the PLP-amino acid adduct and, by specifically enhancing the catalytic efficacy of the cofactor, determines along which of the numerous potential pathways the adduct will proceed. Antibody 15A9 is remarkably reaction- and enantiomer-specific, since no reprotocations other than transamination with D-amino acids and β-elimination of β-chloro-d-alanine were detected. The functional and structural features of 15A9 evince the same chemical necessities of protein-assisted pyridoxal catalysis that were deduced from a comprehensive comparison of the PLP-dependent enzymes (29, 30): the stacking of the PLP ring with hydrophobic residues, the maintenance of the hydroxypyridine moiety in its zwitterionic form by salt bridge and hydrogen bond interactions, the cisoid orientation of the C4’-N bond in the coenzyme amino acid adduct, the strong anchoring of the phosphate group of the cofactor, and the presence of residues facilitating deprotonation and reprotonation in the prototropic shift from aldime 4 to ketimine intermediate 5. The active-site structures and catalytic activities of 15A9 and of mAspAT K258H are consistent with the notion (29) that the covalent imine bond between the aldehyde group of PLP and the ε-amino group of the active-site lysine residue characteristic of PLP-dependent enzymes is not a mechanistic necessity for enzymic transamination. Apart from the lack of covalent binding of PLP, antibody 15A9 with its distinct reaction and enantiomeric specificity and its somewhat limited substrate specificity qualifies by all criteria as a model PLP-dependent protoenzyme (29). To what extent the antibody scaffold will

| Fab 15A9 | kcat (min⁻¹) | kcat (µM⁻¹) |
|----------|--------------|-------------|
| Wild type | 0.33         | 17.5        |
| YH100bf  | 0.03         | 1.1         |
| YH100bK  | 0.10         | 1.7         |
| KH196A   | 0.01         | 8.7         |

*Both wild-type and mutant Fab 15A9 did not catalyze transamination and β-elimination with the L-enantiomers of the substrates at a detectable rate.
allow the improvement of catalytic efficacy by directed evolution remains to be seen.

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