Monoclonal Antibodies against N\textsuperscript{\textalpha}-((5\textprime\prime)-Phosphopyridoxyl)-l-lysine

SCREENING AND SPECTRUM OF PYRIDOXAL 5\textprime-PHOSPHATE-DEPENDENT ACTIVITIES TOWARD AMINO ACIDS

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Cofactors may be expected to expand the range of reactions amenable to antibody-assisted catalysis. The biological importance of pyridoxal 5\textprime-phosphate (PLP) as enzymic cofactor in amino acid metabolism and its catalytic versatility make it an attractive candidate for the generation of cofactor-dependent enzymes. Here we report an efficient procedure to screen antibodies for PLP-dependent catalytic activity and detail the spectrum of catalytic activities found in monoclonal antibodies elicited against N\textsuperscript{\textalpha}-((5\textprime\prime)-phosphopyridoxyl)-l-lysine. This hapten is a nonplanar analog of the planar, resonance-stabilized coenzyme-substrate adducts formed in the PLP-dependent reactions of amino acids. The hapten-binding antibodies were screened for binding of the planar Schiff base formed from PLP and D- or L-norleucine by competition enzyme-linked immunosorbent assay. The Schiff base (external aldimine) is an obligatory intermediate in all PLP-dependent reactions of amino acids. This simple, yet highly discriminating screening step eliminated most of the total 24 hapten-binding antibodies. Three positive clones bound the Schiff base with L-norleucine, two preferred that with the \textalpha-enantiomer. The positive clones were assayed for catalysis of Schiff base formation and of the \textalpha,\textbeta-elimination reaction with the D- and L-enantiomers of \textbeta-chloroalanine. Three antibodies were found to accelerate aldime formation, and two of these catalyzed the PLP-dependent \textalpha,\textbeta-elimination reaction. One of the \textalpha,\textbeta-elimination-positive antibodies catalyzed the transamination reaction with hydrophobic \textalpha-amino acids and oxoacids (Gramatikova, S. I., and Christen, P. (1996) J. Biol. Chem. 271, 30583–30586). All catalytically active antibodies displayed continuous turnover. No PLP-dependent reactions other than aldime formation, \textalpha,\textbeta-elimination of \textbeta-chloroalanine and transamination were detected. The successive screening steps plausibly simulate the functional selection pressures having been operative in the molecular evolution of primordial PLP-dependent protein catalysts to reaction- and substrate-specific enzymes.

The first catalytic antibodies that became known accelerated relatively simple transformations; since then the antibody-catalyzed reactions have increased in complexity and degree of difficulty. Possible strategies to expand the catalytic scope of antibodies include the incorporation of cofactors such as metal ions, heme, thiamine, flavins, nicotinamide, or pyridoxal into the binding sites of the antibodies (1). Pyridoxal 5\textprime-phosphate (PLP)\textsuperscript{1} is probably the most versatile enzymic cofactor. PLP is required by many enzymes that catalyze a wide variety of reactions in the metabolism of amino acids, i.e. transamination, racemization, decarboxylation, aldol cleavage, and elimination and replacement reactions (2). Several attempts to produce pyridoxal-dependent catalytic antibodies have been reported. In the earliest study, a polyclonal antiserum specific for the reduced Schiff base formed from PLP and 3\textprime-amino-l-tyrosine was prepared. The antibodies slightly enhanced the rate of the PLP-catalyzed transamination of l-tyrosine (3–5). A monoclonal antibody against the reduced aldime of pyridoxal and 4\textprime-nitro-l-phenylalanine accelerated aldime formation between 5\textprime-deoxypyridoxal and 4\textprime-nitro-l-phenylalanine but did not catalyze any further reactions (6). Catalysis of Schiff base formation was also observed with a polyclonal antiserum generated against the reduced Schiff base of pyridoxal and d- and l-phenylalanine (7).

The Schiff base 4 generated by condensation of PLP 1 and amino acid 2 (Fig. 1) is the first detectable intermediate common to all nonenzymic and enzymic PLP-dependent reactions of amino acids. In the enzymic reactions, the Schiff base is produced by transamination; the amino group of the substrate replaces the \textepsilon-amino group of the active-site lysine residue which covalently binds PLP in all B6 enzymes. The multiple possibilities for further reactions of the coenzyme-substrate aldime 4 give rise to the diverse PLP-dependent transformations of amino acids (Scheme 1). Reduction of the imine double bond of the aldime by sodium borohydride provides a stable link between the coenzyme and the amino acid. The Ca-nitrogen linkage of the resulting phosphopyridoxyl amino acids 5 (Fig. 1) is similar to that in the tetrahedral carbaminoamine transition state 3 leading to Schiff base formation (8). Phosphopyridoxyl amino acids bind with high affinity to apoenzymes (5, 9, 10) and include all groups important for catalysis with the exception of the imine double bond ensuring the planarity of the Schiff base (Fig. 1). Formation of the planar Schiff base is, however, a prerequisite for the catalytic efficacy of PLP which is due to the electron-withdrawing effect exerted on Ca by the positively charged pyridine nitrogen atom and is mediated through the extensive resonance system of the pyridine ring and the imine double bond.

In a renewed attempt to obtain PLP-dependent antibody

\textsuperscript{1}The abbreviations used are: PLP, pyridoxal 5\textprime-phosphate; B\textalpha enzymes, PLP (vitamin B6)-dependent enzymes; BSA, bovine serum albumin; bis-tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol; ELISA, enzyme-linked immunosorbent assay; FMP, pyridoxamine 5\textprime-phosphate; HPLC, high performance liquid chromatography.
catalysts, we used, as in the previous studies by other laboratories (3-7), a reduced Schiff base as hapten for immunization. The structural disadvantage of this transition state analog was, however, compensated by a special screening protocol. The selection of potential abzymes was based on immunodetection of binders of the Schiff base 4 rather than of the immunizing hapten 5. Binders of the aldime were further screened for α, β-elimination of β-chloroalanine. This easily detectable reaction depends on the deprotonation at Cβ which is an integral step in the by far largest group of PLP-dependent reactions of amino acids (Scheme 1).

EXPERIMENTAL PROCEDURES

Preparation of Hapten, Antigens, Antibody Production and Purification—The synthesis of the hapten 5 and the protein conjugates 6 (Fig. 1) was described previously (3, 11). The hapten N<sup>4</sup>-(5-phosphopyridoxyl)-l-lysine was coupled to maleylated carrier protein (12) which was keyhole limpet hemocyanin or bovine serum albumin (BSA) for immunization and ELISA, respectively. Monoclonal antibodies were generated as described previously (11). The antibodies were purified by affinity chromatography on protein G-Sepharose 4 Fast Flow from Pharmacia Biotech Inc. The concentration of antibody was measured photometrically (\(E_{450,\text{nm}} = 1.4\)).

Screening for Hapten Binding—The hybridoma supernatants were screened by ELISA 10–14 days after the fusion. Maxisorp plates from Nunc were coated with hapten-BSA conjugate 6 (10 μg/ml in 50 mM sodium carbonate, pH 9.6, 50 μg/well) for 1 h at 37 °C, washed with washing buffer (phosphate-buffered saline, 0.05% Tween 20, 0.02% sodium azide), and blocked with BSA (1% w/v in washing buffer, 300 μg/well) for 30 min at 37 °C. The hybridoma supernatants (50 μg/well) were added and the plates incubated for 1 h at 37 °C. The binding of the antigen was detected, after a washing step, with an alkaline phosphatase-labeled second antibody from Sigma. Binders of N<sup>4</sup>-(5-phosphopyridoxyl)-l-lysine were selected by comparison of the binding of hapten-BSA conjugate, maleylated BSA, and unmodified BSA.

Competition ELISA—The inhibitory effects of PLP (0.1–2 μM, depending on the antibody), PLP plus 25 mM D- or L-norleucine, and PLP plus 25 mM glycine on antibody-antigen binding were estimated. Diluted hybridoma supernatant or purified antibodies were preincubated with the inhibitors in bis-tris propane/NaCl (50 mM bis-tris propane, 140 mM NaCl), pH 7.5, at 37 °C for 30 min in the dark. The incubation mixtures were added into the wells that had been coated with antigen (see above), and the plate was incubated for 30 min at 37 °C in the dark. The amount of the bound antibody was measured after addition of an alkaline phosphatase-labeled second antibody from Sigma (for details, see Ref. 11).

Measurement of the Rate of Schiff Base Formation—The purified antibodies and the D- or L-enantiomer of N-ε-acycetyllysine were mixed in bis-tris propane/NaCl, pH 7.5, at 25 °C. The reaction was started by the addition of PLP. The final concentrations of the antibodies, PLP, and the amino acid were 2.5 μM, 16 μM and 1 mM, respectively. The absorbance of the reaction mixture was monitored in the range of 410–450 nm, depending on the antibody, with an HP 8453 spectrophotometer. This wavelength range corresponds to the absorption band of the protonated Schiff base 2 (2, 5).

Determination of α, β-Elimination Activity—The production of pyruvate in the presence of 10 μM antibody, 100 μM PLP, and 10 mM D- or L-enantiomers of β-chloroalanine in bis-tris propane/NaCl, pH 7.5, at 25 °C in the dark was measured with lactate dehydrogenase and NADH. A control reaction without antibody was run under the same conditions. Absorbance at 340 nm was measured with an HP 8453 spectrophotometer. The calculation of all catalytic activities is based on the concentration of binding sites of the antibodies.

Measurements of Tryptone Release—The reaction mixtures (40 μl) contained 25 mM antibody, 1 mM PLP, 25 mM glycine, and 10<sup>6</sup> cpm of [2-<sup>14</sup>]<sup>l</sup>lysine (Isotopchim) with a specific radioactivity of 30 Ci/mmol in bis-tris propane/NaCl, pH 7.0. After an incubation of 1 h at 25 °C in the dark, the released tritium was measured in 10-μl samples as described previously (5).

Determination of Transaminase Activity—The reaction mixtures contained 5–10 μM antibody, 200 μM PLP, and 200 mM L-alanine in bis-tris propane/NaCl, pH 7.5, at 25 °C in the dark. The increase in both absorbance at 325 nm (ε = 8,300 M<sup>-1</sup> cm<sup>-1</sup>; Ref. 13) and fluorescence (excitation wavelength 325 nm, wavelength of maximum emission 389 nm) was used to detect PMP as product of transamination. The negative control without antibody was measured under the same conditions.

In the case of very low activity, detection of catalysis by fluorescence is the method of choice because of its higher sensitivity.

HPLC Analysis for Detection of PLP-Dependent Transformations of Amino Acids—The reaction mixtures contained 25 μM antibody, 0.1–1 mM PLP (depending on the binding affinity of the individual antibodies for the cofactor as estimated by competition ELISA), and 100 mM amino acid in bis-tris propane/NaCl, pH 7.5, at 25 °C in the dark. Samples were taken during a period of 6 h, derivatized with Marfey reagent, and analyzed by reverse phase HPLC (14). Newly generated peaks were identified by comparison with reference substances.

Measurements of Dissociation Equilibrium Constants—The \(K_d\) values of antibody 15A9 for the hapten 5 (Fig. 1), PLP, and PMP were determined by measuring the quenching of the intrinsic fluorescence of the antibody (excitation wavelength 280 nm; wavelength of maximum emission 342 nm). The concentration of the abzyme was in the range of 0.01–0.8 μM. The measurements were performed at 25 °C in bis-tris propane/NaCl, pH 7.5, with a Spex Fluorolog spectrophotometer and were corrected for the fluorescence of the ligand itself. \(K_d\) values were calculated by nonlinear regression analysis.

RESULTS

Screening for Aldimine Binding—Because the imine double bond of Schiff base 4, which is essential for PLP to exert its catalytic effect, was absent in the hapten N<sup>4</sup>-[(5-phosphopyridoxyl)-l-lysine 5 (Fig. 1), we introduced an additional screening step to select from the 24 hapten-binding antibodies those that bind also the planar aldime 4. PLP readily forms Schiff bases with primary amino groups in an uncatalyzed equilibrium reaction. Thus, a competition ELISA of the antibody-antigen binding with PLP and amino acids as inhibitors was used to identify the binders of the Schiff base (Scheme 1). Binders of the aldime were expected to be inhibited more strongly by PLP plus amino acid than by PLP or the amino acid alone. As amino acid ligands for these competitive binding assays we chose D- and L-norleucine and glycine. Antibodies 13B10, 8H4, 15A9, 11C2, and 14G1 showed indeed that their binding to the antigen was inhibited more strongly in the presence of PLP plus 25 mM glycine and PLP plus 25 mM D- or L-norleucine than in the presence of PLP or the amino acid alone (Fig. 2). The inhibition of antibody-antigen binding by the Schiff base 4 formed from PLP plus glycine indicates the existence of a binding site for the amino acid moiety of the hapten, and the difference in inhibition by PLP plus norleucine and PLP plus glycine reflects the contribution of the amino acid side chain to the binding of the Schiff base. In antibodies 13B10, 15A9, and 11C2 the side chain of L-norleucine positively contributed to the binding of the Schiff base. In antibody 14G1, a small positive contribution by the side chain of D-norleucine was observed. In

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**Fig. 1.** Nonenzymatic formation of the planar Schiff base 4 from PLP 1 and an amino acid 2. The nonplanar hapten 5 used in binding studies were N<sup>4</sup>-[(5-phosphopyridoxyl)-Nα-acetyl-l-lysine, N<sup>4</sup>-[(5-phosphopyridoxyl)-l-lysine, N<sup>4</sup>-[(5-phosphopyridoxyl)-3′-amino-l-tyrosine, N<sup>4</sup>-[(5-phosphopyridoxyl)-l-tyrosine, N<sup>4</sup>-[(5-phosphopyridoxyl)-l-ala-nine, and N<sup>4</sup>-[(5-phosphopyridoxyl)-l-alanine. N<sup>4</sup>-[(5-Phosphopyridoxyl)-l-lysine was coupled to maleylated keyhole limpet hemocyanin or BSA and used as antigen 6 for immunization and for ELISA, respectively.
all cases, except for antibody 11C2, the contribution of the side chain of the enantiomeric amino acid was negative. Antibody 8H4 bound the Schiff base with both D- and L-norleucine less tightly than that with glycine. The inhibition profiles of antibodies 5G12 and 6E9 are displayed in Fig. 2 to illustrate the binding properties of the great majority of the antibodies which did not show a significant difference in the inhibition by PLP and by PLP plus amino acids. Apparently, these antibodies cannot accommodate the planar aldimine adduct in their binding site. The inhibition tests were applied to supernatants as well as purified antibodies. The observed differences were negligible.

The immunological test for aldimine binding was validated by the detection of catalysis of aldimine formation by part of the selected antibodies. Purified antibodies 13B10 and 15A9 catalyzed Schiff base formation between PLP and Nε-acetyl-l-lysine, and antibody 8H4 between PLP and Nε-acetyl-d-lysine. The stereospecificity of directly measured aldimine formation thus correlates with the stereospecificity of aldimine binding as assessed by competition ELISA. The best catalyst, antibody 15A9, showed a marked acceleration of the condensation reaction at a PLP concentration of 16 μM, which is considerably below its $K_v$ value of 90 μM (Fig. 3). Under the same conditions, antibodies 8H4 and 13B10 also showed significant rate acceleration. Determination of the initial rate at higher concentrations of PLP and amino acid was not possible because the formation of Schiff base would become too fast to be followed without rapid-kinetics methodology. Antibodies 11C2, 14G1, 5G12, and 6E9 did not catalyze aldimine formation.

**Screening for Deprotonation at Cα**—Deprotonation at Cα of the substrate is an integral step in the reaction pathways of the by far largest group of PLP-dependent reactions of amino acids (Scheme 1). The substrate analog β-chloroalanine has been reported to act as a mechanism-based inhibitor of several B8 enzymes (15). Because of the good leaving group in the β-position, deprotonation at Cα is spontaneously followed by $α,β$-elimination. The resulting aminoacrylate intermediate may react with protein side chains or decompose to chloride, ammonia, and easily detectable pyruvate (Fig. 4). Pyruvate production may thus serve as an indicator of Cα-deprotonation. The potential catalysts, i.e. the aldimine-binding antibodies 13B10, 8H4, 15A9, 11C2, and 14G1, were screened for PLP-dependent activity toward the D- and L-enantiomers of β-chloroalanine (see “Experimental Procedures”). Antibody 15A9 showed $α,β$-elimination activity toward β-chloro-D-alanine ($k_{cat} = 50 \text{ min}^{-1}$), and antibody 13B10 proved active toward β-chloro-L-alanine ($k_{cat} = 5 \text{ min}^{-1}$). Antibodies 8H4, 11C2, 14G1, 5G12, and 6E9 did not show any detectable activity toward either enantiomer. The Nε-acetyl-l-lysine containing hapten 9 (20 μM)
completely inhibited the α,β-elimination reaction of β-chloro-L-alanine catalyzed by antibody 15A9, indicating that the catalytic effect of the antibody is due to its specific binding sites.

Measurement of tritium release from [2-3H]glycine was used to confirm the PLP-dependent antibody 15A9-catalyzed deprotonation at χα (for details, see “Experimental Procedures”). Three control experiments containing PLP plus glycine without protein, PLP plus glycine and unspecific IgG, and antibody 15A9 plus glycine without PLP, were performed. The amount of released tritium in the test sample was corrected for that in the control with PLP plus glycine and corresponded to an estimated value of \( k_{\text{cat}} = 7 \text{ min}^{-1} \), considering an isotope effect of 10 and the half-saturation of the antibody with the substrate glycine.

**Detection of PLP-dependent Amino Acid Transformations—**

The specific absorption and fluorescence properties make PMP completely detectable for the product of the transamination reaction. Antibodies 13B10, 8H4, 14G1, and 11C2 did not accelerate the transamination reaction of PLP and D/L-alanine. Antibodies 13B10, 8H4, 14G1, and 11C2 did not accelerate the formation of a readily detectable product of the transamination reaction.

**Antibody 15A9-catalyzed transamination reaction.**

The absorbance measurements were performed in bis-tris propane/NaCl, pH 7.5, at 25 °C. The concentration of the antibody was 5 μM. The concentration of the cofactor and the substrate D-alanine was 200 μM and 200 mM, respectively. The consumption of the protonated Schiff base was followed at 410 nm and the production of PMP at 325 nm.

**FIG. 4. α,β-Elimination reaction of β-chloroalanine (15).**

**FIG. 5. Antibody 15A9-catalyzed transamination reaction.**

The absorbance measurements were performed in bis-tris propane/NaCl, pH 7.5, at 25 °C. The concentration of the antibody was 5 μM. The concentration of the cofactor and the substrate D-alanine was 200 μM and 200 mM, respectively. The consumption of the protonated Schiff base was followed at 410 nm and the production of PMP at 325 nm.

**FIG. 6. Fluorescence titration of antibody 15A9 with PLP.** The measurements were performed in bis-tris propane/NaCl, pH 7.5, at 25 °C. The concentrations of the cofactor were 20, 40, 53, 70, 90, 106, 140, and 170 μM. For details, see “Experimental Procedures.”

**Dissection of PLP-dependent Amino Acid Transformations—**

The multitude of possible transformations of amino acids (Scheme 1) is a major problem in the design of a screening procedure for PLP-dependent catalytic antibodies. In view of this difficulty, we have devised a protocol that screening for the occurrence of two successive crucial reaction steps rather than for a final product. The first step for which the antibodies were screened was the binding of the PLP-amino acid aldimine 4 (Fig. 1). This selection, easily executed with a competition

**TABLE I**

**Dissociation equilibrium constants of antibody 15A9 for phosphopropidoxyl amino acids and cofactors**

The constants were determined by measurement of quenching of the intrinsic antibody fluorescence at pH 7.5 (see “Experimental Procedures”).

<table>
<thead>
<tr>
<th>Ligand</th>
<th>( K_d ) (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( N^+ )-(5′-Phosphopyridoxyl)-Ne-acetyl-L-lysine ( \alpha )</td>
<td>0.026</td>
</tr>
<tr>
<td>( N^+ )-(5′-Phosphopyridoxyl)-L-lysine ( \alpha )</td>
<td>2</td>
</tr>
<tr>
<td>( N^+ )-(5′-Phosphopyridoxyl)-3′-amino-L-tyrosine ( \alpha )</td>
<td>0.06</td>
</tr>
<tr>
<td>( N^+ )-(5′-Phosphopyridoxyl)-L-tyrosine ( \alpha )</td>
<td>1.7</td>
</tr>
<tr>
<td>( N^+ )-(5′-Phosphopyridoxyl)-L-alanine ( \alpha )</td>
<td>1.4</td>
</tr>
<tr>
<td>( N^+ )-(5′-Phosphopyridoxyl)-D-alanine ( \alpha )</td>
<td>1.5</td>
</tr>
<tr>
<td>PMP( ^\alpha )</td>
<td>90</td>
</tr>
<tr>
<td>PMP( ^\beta )</td>
<td>40</td>
</tr>
</tbody>
</table>

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From Ref. 11. The value of \( K_d \) for PLP in both the transamination reaction with D-alanine and the α,β-elimination reaction with β-chloro-L-alanine was ~90 μM; the value of \( K_d \) for PLP in the competition ELISA (see Fig. 2) was 120 μM.

**DISCUSSION**

The specific absorption and fluorescence properties make PMP completely detectable for the product of the transamination reaction. Antibodies 13B10, 8H4, 14G1, and 11C2 did not accelerate the transamination reaction of PLP and D/L-alanine. Antibody 15A9 was found to catalyze the transamination reaction of PLP with D-alanine (Fig. 5) and other hydrophobic D-amino acids (11). The \( k_{\text{cat}} \) value for transamination with D-alanine was 0.42 min\(^{-1}\), corresponding to a 5,000-fold rate acceleration compared with the catalytic effect of PLP alone (11). Antibody 15A9 has been shown previously by HPLC analysis not to catalyze any reaction of amino acids other than transamination. The

**FIG. 6. Fluorescence titration of antibody 15A9 with PLP.**
ELISA (Fig. 2), was particularly important because we used, as in previous studies by other laboratories (5–7), the reduced and thus nonplanar Schiff base 5 as hapten for the immunization. However, formation of the extended planar resonance system, encompassing the pyridine ring of the coenzyme and the imine double bond, is essential for the cleavage of one of the bonds between Coa and its substritutes (2). In the next screening step, the antibodies were selected for catalysis of the breaking of the Co-H bond, which in most PLP-dependent reactions of amino acids follows the formation of the aldime (Scheme 1). The substrate analog β-chloro-ω-L-alanine provided a simple and generally applicable assay for deprotonation at Ca (Fig. 4). Only antibodies that catalyzed both aldime binding and a-deprotonation were analyzed with HPLC for the generation of specific reaction products from both enantiomers of different amino acids.

The screening procedure clearly defines the requirements that have to be met by an enzyme mimic to catalyze a PLP-dependent transformation of an amino acid (Scheme 1). As a corollary, the successive selection steps plausibly simulate the functional selection pressures that presumably were operative in the molecular evolution of B6 enzymes. Comparison of amino acid sequences has shown that the B6 enzymes are of multiple evolutionary origin (16–18). As required for a PLP-dependent catalytic antibody, the ancestor protein of a B6 enzyme family very likely had to possess a PLP and an amino acid binding site with a geometry that accommodated the planar aldime. Competition ELISA (Fig. 2), as well as the Kd values for hapten determined with antibody 15A9 (Table I), indicated that both the coenzyme and substrate moiety interact with the catalytically active antibodies. Recognition of the amino acid side chain is evident from the enantiomeric specificity of the antibodies in the competition ELISA (Fig. 2), which varies in kind and degree, as well as from the order of preference of amino acids in aldime binding, which invariably was N-acytely-lysine > norleucine > alanine > glycine. Although the immunizing hapten was a derivative of an L-amino acid, two of the five aldime-binding antibodies preferred α-amino acids (Fig. 2). The type of binding of PLP to the antibodies has been examined only in 15A9, the only antibody that catalyzes a transamination of an amino acid other than β-chloroalanine. Antibody 15A9 appears to bind PLP covalently (11). Both experiments with nonenzymatic model systems (19) and the residual activity of mutant PLP-dependent enzymes without active-site lysine residue (20, 21) have indicated that formation of the coenzyme-substrate aldime by transamination rather than de novo formation from PLP and amino acid is not essential for catalysis. The ubiquitous occurrence of the coenzyme-binding lysine residue might reflect a historic trait rather than a mechanistic necessity (16, 22). Covalent binding of PLP probably was the very first step in the molecular evolution of B6 enzymes. Primordial B6 enzymes presumably had to cope with low concentrations of the cofactor. In contrast, at the high concentrations of PLP and amino acid used in our experiments aldime is preformed from unbound PLP and amino acid at a rate fast enough and present in a concentration high enough to serve directly as substrate for the abzymes.

The selection of aldime-binding antibodies was followed by screening for a catalytic effect, i.e. the cleavage of the Coa-H bond of the substrate moiety. In the molecular evolution of B6 enzymes, the successive step after acquiring the capacity of aldime binding may be assumed to have been the development of a catalytic apparatus facilitating the cleavage of one of the bonds between Coa and its substititutes. The easily measured α,β-elimination of β-chloro-D/α-l-alanine (Fig. 4) served to test for Co-deprotonation, which underlies the majority of PLP-dependent reactions of amino acids (Scheme 1). Antibody 13B10 was found to catalyze the α,β-elimination of β-chloro-L-alanine which is consistent with its enantiomeric binding specificity. In contrast, antibody 15A9, which preferably binds the aldime with L-amino acids (Fig. 2), catalyzed both the α,β-elimination and the transamination reaction exclusively with α-amino acids. Apparently, the Coa-H bond of the L-amino acid substrate is directed toward an inert surface region of the antibody (11).

Three reactions were found to be catalyzed by the antibodies: formation of aldime, deprotonation at Ca as reflected by α,β-elimination of β-chloroalanine, and transamination. Catalysis of aldime formation might reflect a favorable relative orientation of bound PLP and amino acid. α,β-Elimination of β-chloroalanine and transamination share one important feature: the crucial reaction steps are proton transfers (Scheme 1). Apparently, in antibody 13B10 and 15A9 acid-base groups are positioned in proximity of Ca and Coa/C4′, respectively. Alternatively, water molecules might have access to these atoms and mediate the proton transfers. With antibody 15A9, transamination is 2 orders of magnitude slower than α,β-elimination, suggesting that reprotonation at C4′ is rate-limiting.

Antibody 15A9 is the only antibody catalyzing the transformation of a natural amino acid. The antibody is remarkably reaction-specific, transamination being the only reaction that is observed. The antibody accelerates the transamination reaction not only of PLP and an amino acid but also in the reverse direction with PMP and an oxoacid as substrates (11). The orientation of the Coa-substitutents relative to the plane of the resonance system of imine and coenzyme together with the presence (and absence) of catalytically effective protein side chains serving as general acid-base groups or modulating the electron reparation in the coenzyme-substrate adduct are thought to determine the reaction specificity in B6 enzymes (23, 24). In contrast to the reaction specificity, the substrate specificity of 15A9 is less strictly defined, apparently all hydrophobic α-amino acids, and oxoacids in the reverse reaction with PMP, are generally accepted as substrates (11). Thus, the results of the successive steps in the functional screening of PLP-dependent antibody catalysts correspond to the molecular evolution of B6 enzymes also with respect to the development of specificity. In the evolution of B6 enzymes, specialization for reaction specificity clearly preceded that for substrate specificity (16, 25). The analogy reflects the interplay of chance and necessity being operative in both cases.

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Screening of Antibodies for PLP-dependent Activity

Monoclonal Antibodies against $N^\alpha$-(5'-Phosphopyridoxyl)-L-lysine: SCREENING AND SPECTRUM OF PYRIDOXAL 5'-PHOSPHATE-DEPENDENT ACTIVITIES TOWARD AMINO ACIDS

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