Coenzymes might efficiently extend the catalytic potential of antibodies. Monoclonal antibodies against N\(^5\)-phosphopyridoxyl-\(l\)-lysine were screened for: 1) binding of \(5'\)-phosphopyridoxyl amino acids, 2) binding of Schiff base of pyridoxal \(5'\)-phosphate (PLP) and amino acids, the first intermediate of all PLP-dependent reactions, and 3) catalysis of PLP-dependent \(\alpha,\beta\)-elimination with \(\beta\)-chlooro-\(d\)/\(l\)-alanine. All three criteria were met by antibody 15A9. Further analysis for PLP-dependent reactions showed that this antibody catalyzes the cofactor-dependent transamination of hydrophobic \(d\)-amino acids and oxo acids (\(k_{cat} = 0.42\) min\(^{-1}\) with \(d\)-alanine). No other reactions with either \(d\) or \(l\)-amino acids were taking place. PLP markedly contributes to catalytic efficiency, being a 10\(^4\) times more efficient acceptor of the amino group than pyruvate. The antibody further accelerates the reaction \(k_{cat(antibody)}/k_{cat(PLP)} = 5 \times 10^{3}\) with \(d\)-alanine as substrate) and ensures reaction specificity, stereospecificity, as well as limited substrate specificity.

Nonproteinaceous cofactors endow many enzymes with chemical functions that their protein parts alone cannot provide. Thus, the combination of a binding-specific antibody with a reactive cofactor seems a promising approach to extend the range of reactions that can be catalyzed by antibodies. Pyridoxal \(5'\)-phosphate (PLP), a derivative of vitamin B\(_6\), serves as prosthetic group of enzymes that catalyze manifold transformations of amino acids, i.e. transamination, racemization, decarboxylation, aldol cleavage, elimination, and replacement reactions (1). In the earliest attempt to generate catalytically active antibodies, a polyclonal antiserum against the reduced Schiff base formed from PLP and \(3'\)-amino-\(l\)-tyrosine was prepared. The antibodies were found to bind the amino acid, PLP, and their aldmine adduct. They brought about, in comparison to PLP alone, a 5 times enhanced rate of \(\alpha\)-proton exchange of \(l\)-tyrosine (2). A monoclonal antibody, generated against the reduced aldmine of pyridoxal and 4'-nitro-\(l\)-phenylalanine, accelerated aldmine formation between 5'-deoxypyridoxal and 4'-nitro-\(d\)-phenylalanine. However, the antibody did not promote any further reactions characteristic of pyridoxal catalysis (3).

A major problem in producing PLP-dependent enzymes is the design of transition state analogs of the covalent coenzyme-substrate adducts to be used as hapten. The planar aldmine intermediate 3 (Fig. 1) formed from PLP and the amino acid substrate 2 is common to all enzymatic and nonenzymatic PLP-dependent reactions of amino acids. The electron-withdrawing effect of the extended resonance system of the imine double bond and the positively charged pyridine ring labilizes the bonds to the substituents of Ca (1, 4). The bond cleaved at Ca and the subsequent covalency changes determine which of the possible transformations of the amino acid is executed. A stable analog of aldmine intermediate 3 is readily obtained by reduction of the imine double bond. The resulting N\(^{\text{\text{*}}}\)-(5'-phosphopyridoxyl) amino acids bind tightly to the apoproteins of the corresponding PLP-dependent enzymes (2, 5, 6), include all groups of both coenzyme and substrate that participate in binding and catalysis, except the imino group, and have proven reliable analogs of the covalent coenzyme-substrate adducts in x-ray crystallographic studies (4). Their only, but serious disadvantage, if used for eliciting catalytic antibodies, is the lack of the imine double bond which abolishes the planarity of the compound. The formation of the planar resonance system of the aldmine intermediate 3, however, is essential for the catalytic effect of the cofactor.

Here we report the first successful attempt to generate PLP-dependent catalytic antibodies. The use of a phosphopyridoxyl amino acid as a suboptimum hapten was offset by an additional screening step.

**EXPERIMENTAL PROCEDURES**

**Preparation of Haptenes and Antigens—**Pyridoxal \(5'\)-phosphate (1 mmol), \(l\)-lysine (1.5 mmol), and KOH (7 mmol) were dissolved in 10 ml of methanol, and portions of solid NaBH\(_4\) were added until the solution was decolorized (6). The solution was brought to pH 5.5 with concentrated hydrochloric acid. The reduced aldmine was isolated by ion-exchange chromatography (Amberlite CG-50) and high performance liquid chromatography (RP-8). N\(^{\text{\text{*}}}\)-(5'-Phosphopyridoxyl)-N\(^{\text{\text{*}}}\)-acetyl-\(l\)-lysine, N\(^{\text{\text{*}}}\)-(5'-phosphopyridoxyl)-\(l\)-alanine, and N\(^{\text{\text{*}}}\)-(5'-phosphopyridoxyl)-\(d\)-alanine were prepared in the same way. The molecular masses of the haptenes were confirmed by mass spectrometry. Bovine serum albumin and keyhole limpet hemocyanin were maleylated as described (7) and conjugated (2) with N\(^{\text{\text{*}}}\)-(5'-phosphopyridoxyl)-\(l\)-lysine (Fig. 2, 8). After dialysis against phosphate-buffered saline, pH 7.2, the hapten densities (14 molecules hapten/molecule of serum albumin and 10 molecules hapten/functional unit of hemocyanin) were determined by measurement of absorbance at 325 nm due to the phosphopyridoxyl part of the hapten.

**Preparation and Purification of Antibodies—**BALB/c mice were immunized with the reduced Schiff base of PLP and \(l\)-lysine coupled to maleylated keyhole limpet hemocyanin and hybridomas were obtained by standard methods (8, 9). The antibodies from cell culture supernatant were purified by affinity chromatography on Protein G-Sepharose 4 Fast Flow from Pharmacia according to the instructions provided by the manufacturer.

**Competition ELISA—**Both hybridoma supernatants and purified antibodies were tested; the differences were negligible. The measurements were performed in 50 mm Bis-tris propane, 140 mm NaCl, pH 7.5. Prior to carrying out competition ELISA, optimum antibody concentration and incubation time for establishing a titration curve were determined. Maxisorp plates from Nunc were coated with bovine serum albumin-hapten conjugate (50 \(\mu\)l per well, 10 \(\mu\)g/ml in 50 mm sodium carbonate buffer) / 30583
RESULTS AND DISCUSSION

The antibodies were first screened by ELISA for binding of N⁴(5'-phosphopyridoxyl)-L-lysine coupled to bovine serum albumin (Fig. 2). Thirty IgG-producing hybridomas were selected. Antibodies that bound to the carrier protein plus spacer without hapten were eliminated. PLP-binding antibodies were then selected by competition ELISA in which PLP competed with the antigen for binding to the antibody (Scheme 1). The next screening step was crucial: it compensated the use of nonplanar N⁴(5'-phosphopyridoxyl)-L-lysine as hapten in the immunization. The following criterion was applied to select by competition ELISA potential binders of the planar PLP-amino acid aldimine: the difference in inhibition of the antibody-fluorescence (excitation wavelength 280 nm, emission wavelength 342 nm).

The remaining five potential catalysts were screened for PLP-dependent catalytic activity toward the D- and L-amino acids. Due to its good leaving group in the β-position, this substrate analog is decomposed to chloride, ammonia, and readily detectable pyruvate in an α,β-elimination reaction that is initiated by deprotonation of Ca (10). β-Chloroalanine thus allows a convenient and almost universal screening for deprotonation at Ca which is an integral step in most PLP-dependent reactions of amino acids (1). Two antibodies proved active toward β-chloro-D/L-alanine (Scheme 1). Due to its higher activity, antibody 15A9, which was active toward the D-enantiomer, was chosen for further exploration.

Spectrophotometric testing of antibody 15A9 for reactivity toward other D- and L-amino acids showed that it catalyzed the transamination reaction between hydrophobic D-amino acids and PLP (Table I). In the α,β-elimination reaction of β-chloro-D-alanine, PLP acts as true cofactor that does not partake in the stoichiometry of the reaction. In the transamination half-reaction (e.g. D-alanine + PLP → pyruvate + PMP), however, PLP serves as a second substrate; amino acid and PLP are continuously transformed to the products oxoaacid and PMP until the reaction equilibrium is reached. The abzyme 15A9 was further screened with an analytical protocol that allows to detect both the consumption of any amino acid substrate and the formation of any products, including enantiomers, provided they carry a primary amino group (11). Samples of the reaction mixture containing 25 μM antibody, 1 mM PLP, and 1 mM amino acid were derivatized with Marfey reagent and analyzed by high performance liquid chromatography. None of the potential PLP-dependent reactions other than transamination was found to take place. Only D-enantiomers of preferably aliphatic hydrophobic amino acids were accepted as substrates. The pH-rate profile of the 15A9-catalyzed half-reaction of transamination with D-alanine had an optimum at pH 7.5. Antibody 15A9 also catalyzed the reverse half-reaction of transamination, i.e. the conversion of pyruvate or 2-oxocaproate to D-alanine or D-norleucine, respectively, in the presence of PMP with concomitant production of PLP (Table I). The two half-reactions could be joined to result in a complete transamination cycle, i.e. antibody 15A9 together with the cofactor catalyzed the transformation of D-norleucine and pyruvate to 2-oxocaproate and D-alanine.

Control experiments ruled out that the observed catalytic effects were due to contaminating enzymes from the hybridoma cells. Other antibodies against the same antigen and obtained by the same procedure did not catalyze the α,β-elimination reaction of β-chloro-D/L-ala.; reduction with NaBH₄ in the presence of PLP (see below) only partially destroyed the catalytic activity (PLP-dependent enzymes would be completely and irreversibly inactivated by reduction of the imine bond between PLP and the active-site lysine residue). Furthermore, in the antibody-catalyzed reactions, PLP serves as a dissociable cofactor rather than as a tightly bound prosthetic group; and last, no enzymes for D-amino acids are known to occur in mammals. The antibody-catalyzed α,β-elimination reaction of β-chloro-D-alanine was completely inhibited by 20 μM N⁴-
acetyl-L-lysine containing hapten 9 indicating that the catalytic effect is due to the specific binding site of the antibody.

Antibody 15A9 probably binds PLP solely by noncovalent interactions. Noncovalent binding of PLP is indicated by the similar $K_d$ values for PLP and PMP, 90 and 40 $\mu M$, respectively. The $K_d$ for PLP agrees with its $K_m$ value in the half-reaction of transamination with $d$-alanine. In agreement with noncovalent binding, i.e. the absence of an imine bond between PLP and an $\epsilon$-amino group of the antibody, the catalytic activity was not abolished by reduction with borohydride. The antibody (10 $\mu M$) and 200 $\mu M$ PLP were incubated in the dark for 5 min at pH 8.5 and 25 °C; after reduction with 2 $\mu M$ NaBH$_4$ (20 min) and buffer exchange, half of the transaminase activity of the antibody was recovered. As another characteristic of antibody-catalyzed transamination, aldime 3 (Fig. 1), is preformed in solution rather than generated by transamination as in the enzymic reaction. Under the conditions used for following antibody-catalyzed transamination (see legend of Table I), the uncatalyzed formation of the Schiff base is completed within seconds. After binding to the antibody, the aldime intermediate is deprotonated at C4 to give the ketimine intermediate 5. The absorption spectrum of the antibody in the presence of $d$-alanine plus PLP or pyruvate plus PMP did not show a band at 490 nm that would indicate the formation of the quinonoid intermediate. The absence of a spectroscopically detectable quinonoid intermediate might be due to kinetic reasons or, less likely, to a concerted mechanism of the aldime-ketimine tautomerization. The last step, hydrolysis of ketimine 5 to PMP 6 and oxocarboxy group 7, corresponds to a reversal of Schiff base formation. Model reactions indicate that ketimine hydrolysis is too fast to be kinetically significant in the antibody-catalyzed reaction. The absence of racemization of any of the tested amino acids indicates that proton exchange at C4 (Step 3 $\Rightarrow$ 4) occurs stereospecifically (Fig. 1). Antibody 15A9 has been elicited with the L-lysine-containing antigen 8 (Fig. 2). The $N^\epsilon$-acetyl-L-lysine-containing hapten is bound very tightly with $K_d = 26 \text{ nM}$. The L-ala-nine-containing hapten is bound considerably less tightly ($K_d = 1.4 \text{ $\mu M}$) and is only slightly more strongly bound than its enantiomer ($K_d = 1.5 \text{ $\mu M$}$). The preference for the L-enantiomer is more pronounced in the binding of the Schiff base of PLP with norleucine (Fig. 3). Catalytic activity, however, is observed exclusively with $d$-amino acids (Table I). The discrepancy suggests that the Co-H bond of L-amino acid aldimes points toward a chemically inert surface region of the antibody while with $d$-amino acids deprotonation at Co is assisted either by an acid/base group of the antibody or by a water molecule.

The comparison of the rates of both $\alpha,\beta$-elimination and transamination in the absence of cofactor with the rates of the
For both α,β-elimination and transamination, the first-order rate constants of the reactions of the indicated species are compared. The first-order relative rate constants were calculated from the \( k_{\text{cat}} \) values given in Table I, if not indicated otherwise. All rates were measured at 25 °C with the exception of that of ε-norleucine and pyruvate (see Footnote d). The two entries at the bottom of the Table serve to estimate the contribution of PLP to catalysis of the transamination reaction; the second-order rate of the production of alanine from pyruvate and an amino acid is compared with the second-order rate of the reaction of PLP with an amino acid. AspAT is included in the comparison as a prototype PLP-dependent enzyme (12).

| Reaction | Reactive species | Relative rate constants |
|----------|------------------|------------------------|
| α,β-Elimination | β-Chloro-δ-alanine<sup>a</sup> | 1 |
| | ε-Chloro-δ-alanine-PLP | 10⁴ |
| | β-Chloro-δ-alanine-PLP · Ab | 2 × 10⁷ |
| | β-Chloro-δ-alanine-PLP · AspAT<sup>b</sup> | 1.2 × 10⁸ |
| Transamination | ε-Alanine-PLP | 1 |
| | ε-Alanine-PLP · Ab | 5 × 10³ |
| | ε-Alanine-PLP · AspAT<sup>c</sup> | 2 × 10³ |
| | ε-Norleucine plus pyruvate<sup>d</sup> | 1 |
| | ε-Alanine plus PLP | 10⁴ |

<sup>a</sup> The rate of the spontaneous α,β-elimination of β-chloroalanine (1 m) was measured.

<sup>b</sup> From Ref. 12.

<sup>c</sup> The rate of transamination between 20 mM ε-norleucine and 40 mM pyruvate was determined under anaerobic conditions at 80 °C for 10 h. The products were analyzed by HPLC (11). Assuming a temperature coefficient of 2, the rate was compared with the rate of transamination with PLP at 25 °C.

Cofactor and antibody effectively complement each other. As in the enzymes, the protein enhances the catalytic efficacy of the cofactor and ensures reaction specificity, stereospecificity, and substrate specificity. Natural and custom-tailored artificial cofactors, even if dissociable, may thus extend the catalytic space attainable by antibodies.

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Paraphrasing the first-order rates of the reactions of the amino acid-PLP aldimines. The rate acceleration brought about by the antibody is similar for α,β-elimination of β-chloroalanine and transamination, i.e. 2 × 10⁴ and 5 × 10³, respectively (Table II). The catalytic effect of the cofactor is easy to assess in the case of the monomolecular α,β-elimination reaction. For the bimolecular transamination reaction, an estimate may be obtained by comparing the efficacy of PLP and an oxoacid as acceptor of the amino group from an amino acid. The reactions of amino acids with oxoacids are very slow and had to be performed at high temperature. Remarkably, the estimated rate at 25 °C coincides with the acceleration factor of 10⁴ obtained for α,β-elimination.

The rate of the spontaneous α,β-elimination of β-chloroalanine (1 M) was measured.