Antibodies Can Recognize the Chiral Center of Free α-Amino Acids

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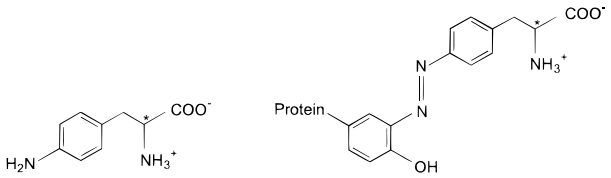
The central importance of stereochemistry in all fields of chemical, biological, and pharmaceutical sciences, both for theoretical research as well as in practical applications, continues unabated. This includes the development, investigation, and production of enantiomerically pure drugs and other compounds by asymmetric synthesis, diastereomeric and enzymatic resolution, and enantioselective chromatography.[1] Currently available techniques for enantiomer and diastereomer recognition are empirical and not general. Since it is possible to raise antibodies against virtually any compound, it is surprising that antibody-based approaches have only rarely been used for the detection[2] and resolution[3] of chiral compounds. Here, we describe novel, tailor-made "class specific" antibodies, both anti-D-α-amino acid antibodies and anti-L-α-amino acid antibodies, which display high chiral recognition but relaxed specificity for the side chain. Such antibodies can therefore be used for isolation and configuration assignment of α-amino acids. Our results also suggest a new and general approach for resolution and configuration assignment of hitherto unresolved chiral compounds of other classes of substances and for the determination of enantiomeric ratios.

The α-amino acids represent one of the most important classes of substances in nature which possess a stereogenic center and, therefore, exemplify a good system to demonstrate the applicability of antibodies to address stereochemical problems. Although antibodies were raised against amino acid derivatives in the classic studies of Landsteiner and others,[4] these authors did not examine antibody binding by *free* amino acids. More recent studies of antibody binding to amino acids such as L-glutamic acid and L-aspartic acid were stimulated by the desire to detect these and other neurotransmitters by immunocytochemistry;[5] antibodies produced were specific for the individual amino acid used as hapten[6] and sometimes displayed enantiomeric specificity.[7] Fuji and co-workers reported the production of antibodies catalyzing the hydrolysis of *N*-(carbobenzoxy)-amino acid esters;[8] using a racemic phosphonate transition state analogue for immunization, they screened and selected monoclonal antibodies that enantioselectively hydrolyze amino acid esters possessing various α-substituents.

To demonstrate that stereospecific antibodies could be directly raised against a broad class of substances, such as the α-amino acids, we linked a protein carrier to an enantiomerically pure amino acid hapten distal to the α-amino acid function, the carboxylate/primary amino/hydrogen triad, so as to expose this functional grouping for maximal antibody binding. Therefore, *p*-amino-D- and L-phenylalanine (D-**1** and L-**1**), respectively, were coupled to keyhole limpet hemocyanin (KLH) via the *p*-amino group by diazotization of the tyrosyl residues,[9] and the resulting conjugates, *p*-azo-D-phenylalanine-KLH (D-**2**) and *p*-azo-L-phenylalanine-KLH (L-**2**), were used to immunize rabbits. Enantiospecific rabbit antibodies against D-**2** (anti-D-AA) and against L-**2** (anti-L-AA), respectively, were detected in an enzyme-linked immunosorbent assay (ELISA) by antibody binding to solid-phase bound conjugates of D-**1** or L-**1** and bovine serum albumin (BSA) prepared by diazotization (D-**3**, L-**3**).[10] Both antibodies, anti-D-AA and anti-L-AA, specifically bind to the hapten-congruent BSA conjugates D-**3** and L-**3**, respectively, while no significant binding to the opposite enantiomers is observed.



1 **2** Protein = KLH
3 Protein = BSA

The specificity and relative affinity of the antibodies for the enantiomers of free amino acids was determined by inhibition of antibody binding to solid-phase bound D-**3** or L-**3**.[11] Binding of anti-D-AA and anti-L-AA could be inhibited by micro- to millimolar concentrations of the corresponding enantiomers of free amino acids. The free amino acids *p*-aminophenylalanine and phenylalanine, whose structures overlap with the hapten, were found to be strong inhibitors and were recognized enantiospecifically by the corresponding antibodies (Figure 1). Furthermore,

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(10) The end-point titers of total sera obtained were 1:>500000 for anti-D-AA and 1:>250000 for anti-L-AA, respectively. There was no detectable recognition of BSA alone. For further use, antibodies were enriched by precipitation with ammonium sulfate and dialyzed against phosphate-buffered saline to give final concentrations of 19.3 mg/mL (anti-D-AA) and 21.2 mg/mL (anti-L-AA).

(11) (a) In competitive assays, ELISA plates were coated with D-**3** or L-**3**. Antibodies were incubated together with free amino acids in varying concentrations as inhibitor. Inhibition of antibody binding to the solid phase was calculated using the equation % inhibition $= (1 - (A/A_0)) \times 100$, where A represents absorbance values in the presence of inhibitor, while A_0 is the absorbance obtained without inhibitor. The concentration of each amino acid necessary to inhibit the binding of antibody to immobilized conjugates by 50% (I_{50}) was determined by interpolation assuming linearity near the point of 50% inhibition. (b) Competitive ELISA procedures provide *relative affinities* rather than *real affinities*. For a critical discussion on the determination of affinities by ELISA, see: Goldberg, M. E.; Djavadi-Ohaniance, L. *Curr. Opin. Immunol.* **1993**, *5*, 278−281. Underwood, P. A. *J. Immunol. Methods* **1993**, *164*, 119−130.

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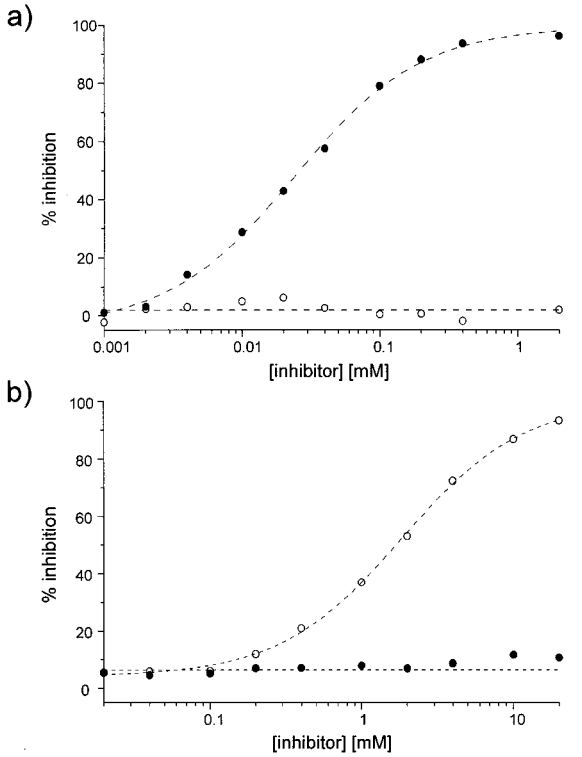

Figure 1. Inhibition of binding of anti-D-AA to immobilized D-**3** (a) and of anti-L-AA to L-**3** (b) by varying concentrations of free D-phenylalanine (●) and L-phenylalanine (○).

enantiomeric specificity is exhibited for other amino acids, aromatic and aliphatic, proteinogenic and nonproteinogenic (Table 1). As the amino acid becomes less like the hapten in side group structure, higher concentrations are required to detect antibody binding, but the enantiomeric specificity is still clearly observed even with amino acids that are far different in their side chains from the immunizing hapten, e.g., N^{ϵ}-DNP-lysine.[12] The order of binding follows what would be expected from the decreasing structural analogy of inhibitor with the immunizing hapten.[13] In general, due to the lower affinity of the anti-L-AA antibodies compared to the anti-D-AA antibodies, higher concentrations of the free amino acids were required for inhibition in the competitive tests with anti-L-AA. Amino acids which lack the α-amino acid carboxylate/primary amino/hydrogen triad, such as proline, β-alanine, and ϵ-aminocaproic acid, were not bound by the antibodies under the conditions used; glycine, too, was not bound.

The ubiquitous presence of free amino acids in blood and tissue might have been expected to preclude the formation of tight and selective antibody binding to this class of substances. However, high specificity antibodies for D- or L-α-amino acids can easily be obtained from simple anti-hapten rabbit sera. An immediate ramification of these results is the use of such antibodies for the isolation and purification of amino acids in affinity chromatography as well as easy, rapid configuration assignment to very small amounts of α-amino acids without derivatization.[14] Hundreds of α-amino acids have been isolated from biological and nonbiological samples,[15] and there is a wider prevalence of D-amino acids and D-amino acid peptides than is generally assumed, even in human tissue,[16] but aside from X-ray crystallographic methods, which require appreciable and crystallizable quantities of material, no entirely applicable methods for assigning absolute configuration to amino acids, or other chiral compounds, are available. Suitably raised antibodies such as those described here may not only be used for this purpose but also for the study of other challenging problems of chiral recognition.

Table 1. Relative Affinities of Antibodies for Free α-Amino Acids

competitive amino acid	I_{50} [mM][a]
anti D-AA	
D-tyrosine	0.010 ± 0.001
p-amino-D-phenylalanine	0.023 ± 0.003
D-phenylalanine	0.025 ± 0.001
D-tryptophan	0.049 ± 0.001
D-cyclohexylalanine	0.16 ± 0.01
N^{ϵ}-DNP-D-lysine	0.25 ± 0.03
D-histidine	1.39 ± 0.05
D-norleucine	2.26 ± 0.12
D-leucine	4.80 ± 0.27
D-valine	15.9 ± 0.8
anti-L-AA	
p-amino-L-phenylalanine	1.06± 0.10
L-phenylalanine	1.63 ± 0.07
L-tryptophan	8.79 ± 0.53
L-histidine	38.0 ± 5.0
L-norleucine	52.8 ± 4.6

[a] Concentrations of free amino acids necessary to inhibit binding of anti-D-AA and anti-L-AA to immobilized D-**3** and L-**3**, respectively, by 50% (I_{50}) were determined by competitive ELISA. In each case, no inhibition by the opposite enantiomer was detected using up to 200-fold higher concentrations than the I_{50} values given. The maximum concentration usable was limited by the solubility of the amino acid.

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Supporting Information Available: Experimental details for the preparation of hapten−protein conjugates and antisera, ELISA protocols, and graphical illustration of the noncompetitive ELISA results (3 pages, print/PDF). See any current masthead page for ordering information and Web access instructions.

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(12) We found group-selective specificity for the α-amino acid stereogenic center using a total of 31 amino acids with a different, more sensitive assay (unpublished results).

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(14) Preliminary results also suggest that these antibodies may be used for quantification of enantiomeric purity, especially at the outer limits of current methods for estimating enantiomer ratios. Antibody anti-D-AA has been successfully used for this purpose, and the results will be reported separately.

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