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

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Conjugate preparation. D-**1** or L-**1** (1 mmol) was dissolved in 16.3 ml of 0.1 N hydrochloric acid. Equimolar amounts of sodium nitrite were carefully added to the cooled solution with continuous stirring. After 10 minutes, the solution was added dropwise to the protein (10 mg/ml) dissolved in 100 mM sodium tetraborate, pH 8.5. The amount of diazo reagent added was twofold to the quantity of tyrosine and histidine residues in the protein. During the addition the pH was maintained by adding 0.1 N NaOH. Coupling was allowed to proceed at 4°C overnight. Afterwards, the azoproteins were dialysed extensively against phosphate buffered saline (PBS), pH 7.4. The hapten densities were estimated using the extinction coefficients at 460 and 500 nm (Tabachnick, M.; Sobotka, H. *J. Biol. Chem.* **1960**, 235, 1051-1054) and were found to be: D-**2**, 8 residues per functional unit (MW 51,000); L-**2**, 11; D-**3**, 8 residues per molecule (MW 67,000); L-**3**, 9.

Antisera preparation. D-**2** or L-**2** (100 μg) was mixed with complete Freunds adjuvant and subcutaneously injected on the backs of 12 week old male New Zealand white rabbits. Sera were obtained after booster injections with 100 μg antigen with incomplete Freunds adjuvant two, four and eight weeks after the first injection. The titer of enantiospecific immunoglobulins in sera was determined by noncompetitive ELISA using D-**3** and L-**3** as coating. The imunoglobulins were enriched for further use by precipitation with 45% ammonium sulfate. After dialysis against PBS, protein concentrations were determined by

UV absorption at 280 nm and found to be: 19.3 mg/ml for anti-D-AA and 21.2 mg/ml for anti-L-AA. Aliquots of antibodies were stored at -20°C until use.

ELISA. A noncompetitive ELISA procedure was used to detect antibodies with stereospecific binding to solid phase bound D-**3** or L-**3** (Fig. 1). Microtiter plates were coated with 100 µl per well of 1 µg/ml D-**3** or L-**3** in 50 mM carbonate buffer, pH 9.6, overnight at 4°C. One third of the wells was incubated with 100 µl/well incubation buffer, as a control. The plates were washed three times with PBS containing 0.05% Tween-20 (PBS/Tween) and blocked by incubating with 250 µl per well PBS/Tween containing 1% gelatin. After 2 h incubation at 37°C, the plates were washed three times with PBS/Tween and serial dilutions of antibody in PBS were added in triplicate (100 µl/well). Following incubation for 2 h at 37°C the plates were washed three times with PBS/Tween and then 100 µl/well of horseradish peroxidase-conjugated goat anti-rabbit $F(ab')_2$ in PBS (1:10,000) were added. After 2 h incubation at 37°C, the plates were washed three times with PBS/Tween and 100 µl/well of substrate solution was added. After 5 min, 1 N sulfuric acid, 100 µl/well, was added as stopping solution and the absorbance was read in a plate reader at 492 nm.

A competitive ELISA procedure was used to test specificity and relative affinity of the antibodies for the enantiomers of free underivatized amino acids. Microtiter plates were coated with 1 µg/ml D-**3** or L-**3** (100 µl/well) in 50 mM carbonate buffer, pH 9.6, overnight at 4°C. After washing and blocking (as described for the noncompetitive ELISA), 50 µl/well of serial dilutions of the pure enantiomers of free amino acids in PBS were added in triplicate. Antibody, 50 µl, in a fixed dilution was added to each well. Taking into account the 1:2 dilution in this step, the effective dilution in the tests were 1:100,000 for the anti-D-AA and 1:25,000 for the anti-L-AA. The remaining procedure was the same as described for the noncompetitive ELISA.

Absorbance values (A) were converted into % inhibition values using the equation: % inhibition = $(1 - (A/A_0))x100$. A_0 values were the average of at least 8 wells without competitive amino acid. The concentration of each amino acid necessary to inhibit the binding of antibody to immobilized hapten-BSA conjugate by 50% (I_{50}) was determined by

interpolation assuming linearity near the point of 50% inhibition. The I_{50} value for each amino acid represents the average of at least three separate assays.

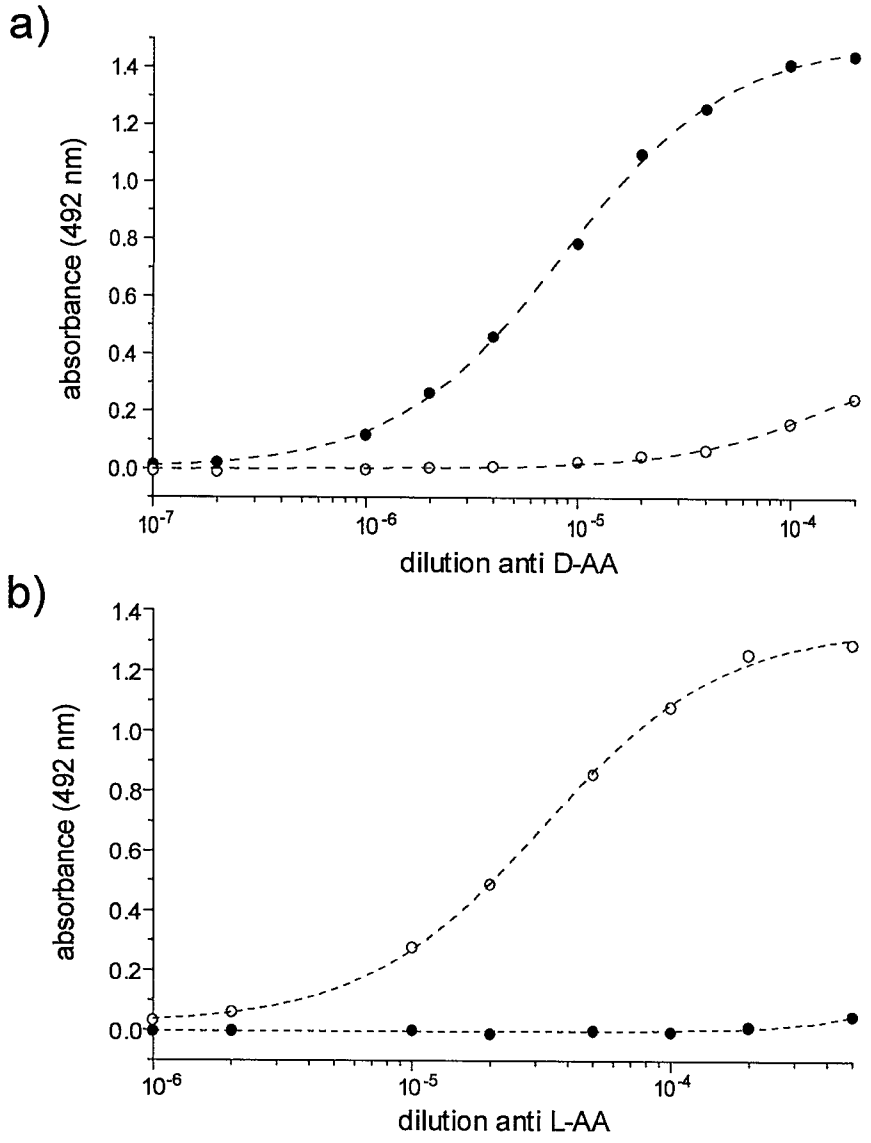

Fig. 1. Binding of anti-D-AA (a) and anti-L-AA (b) to D-3 (•) and L-3 (o). ELISA plates were coated with 1 μg/ml of D-3 or L-3; 100 μl per well of antibodies in serial dilutions were added.