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Specific recognition of a tetrahedral phosphonamidate transition state analogue group by a recombinant antibody Fab fragment

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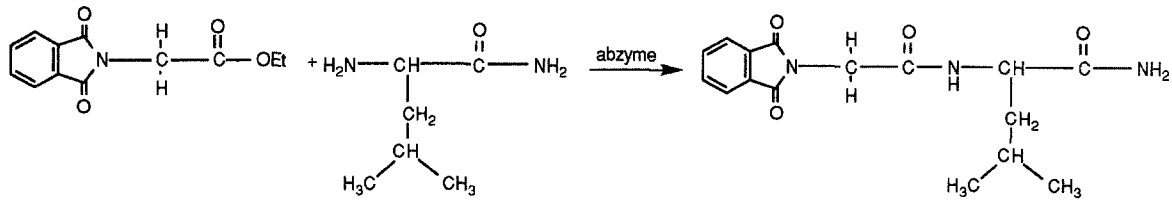
Summary. In order to obtain antibodies able to catalyse a peptide synthesis, a naive combinatorial library of human Fab antibody fragments was screened with the phosphonamidate transition state analogue of the reaction. Several Fab fragments were able to bind the analogue. Competitive binding studies performed with molecules containing representative parts of the hapten showed that two Fabs were able to recognize specifically the tetrahedral phosphorus present in the hapten.

Keywords: Amino acids – Phosphonamidate – Transition state – Combinatorial library

Introduction

In 1986, the Lerner and Schultz research groups reported the production of antibodies with enzyme-like activities (Tramontano, 1986; Pollack, 1986). These catalytic antibodies (or abzymes) were raised against a stable transition state analogue of the catalysed reaction.

Our goal is to produce antibodies displaying catalytic activities in peptide synthesis (Scheme 1). The chemical synthesis of large peptides based on small peptide fragments coupling is still under-developed because of the risk of racemisation and the need for side chain protection. Recently, some chemoselective methods such as chemical ligation have been developed but peptide coupling either occurs at very specific residues or yields chimeric proteins (Gaertner, 1992; Liu, 1994; Dawson, 1994). On the other hand, the enzymatic synthesis of peptides is limited usually to the coupling of L-amino acids only (Jakubke, 1995). Antibody-catalysis would provide a new method for peptide (Hirschmann, 1994; Jacobsen, 1994) and pseudopeptide coupling.



Scheme 1. Model study for Glycine-Leucine coupling reaction catalysed by antibody

We used a phosphonamidate structure **H** as the transition state analogue of the coupling reaction between glycine and leucine (Scheme 2). After coupling to a carrier protein, we screened 6.5×10^{10} different Fab fragments of a naive human combinatorial library (Griffiths, 1994). We selected 6 different Fab fragments able to recognise the soluble hapten. We were particularly interested in the selection of antibodies directed specifically against the tetrahedral phosphonamidate group which mimics the transition state of the reaction.

Material and methods

*Experimental procedure for the preparation of hapten **H** and competitor **C_2***

Hapten **H**

PCl_5 (1.25 g, 6 mmol) was added to phtalimidomethyl diethylphosphonate (1.48 g, 5 mmol) in 20 mL of C_6H_6. The reaction mixture was refluxed overnight then evaporated. CH_2Cl_2 was added then evaporated and this was repeated twice. Then 10 mL of CH_2Cl_2 was added and to this solution was added dropwise a solution made of N-Leucyl-6-amino caproic acid methyl ester hydrochloride (1.47 g, 5 mmol), NEt_3 (2.0 g, 20 mmol) and 20 mL of CH_2Cl_2. The reaction mixture was stirred overnight at room temperature. The CH_2Cl_2 was then evaporated. AcOEt was added, the precipitate of NEt_3HCl was filtered off and this was repeated three times. Et_2O was added and a grey precipitate was obtained. Filtration yielded 1.05 g (41%) of the title compound as a mixture of diastereoisomers: MP 118–122°C; ^{1}H NMR ($CDCl_3$, Me_4Si) δ 0.7–0.95 (m, 6H), 1.1–1.75 (m, 12H), 2.25 (t, I = 7.5 Hz, 2H), 2.9–3.3 (m, 2H), 3.62 (s, 3H), 3.65–4.15 (m, 5H), 6.63 (t, I = 5 Hz, 1H), 7.6–7.9 (m, 4H); ^{31}P NMR ($CDCl_3$, H_3PO_4) δ 23.59; MS(FAB) m/e = 510 (M+1), 532 (M+23). High resolution MS(FAB) calcd for $C_{24}H_{37}N_3O_7P$ (M+1): 510.2369. Found: 510.2306.

Competitor **C2**

This compound was prepared using the same procedure as for the preparation of hapten **H** using methyl diethylphosphonate (0.76 g, 5 mmol) and leucine amide hydrochloride (0.83 g, 5 mmol) to yield 0.40 g (34%) of the title compound as mixture of diastereoisomers: ^{1}H NMR ($CDCl_3$, Me_4Si) δ 0.8–1.0 (m, 6H), 1.27 (d, I = 7 Hz, 3H), 1.41 (d, I = 12 Hz, 3H), 1.5–1.7 (m, 3H), 3.4–4.1 (m, 3H), 5.63 & 5.68 (bs, 2H), 6.53 & 7.23 (bs, 1H); ^{31}P NMR ($CDCl_3$, H_3PO_4) δ 32.87; MS(FAB) m/e = 237 (M+1). High resolution MS (FAB) calcd for $C_9H_{22}N_2O_3P$ (M+1): 237.1368. Found: 237.1373.

Preparation of the peptide-protein conjugate for the ELISA test

After saponification, the hapten **H** was coupled via its free carboxylic group to lysine residues of thyroglobulin (Tg). For this purpose hapten (82 μmol) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) were dissolved in 0.5 ml of phosphate buffer

(0.1 M, pH 7). The reaction was stirred for 30 min at 0°C. To the mixture was added thyroglobulin (20 mg), which had been previously dissolved in 10 μl of DMF. The reaction was stirred overnight at 4°C. Hapten-Tg conjugate was purified by chromatography on Sephadex G50 in water. An identical protocol was performed to couple hapten to Bovin Serum Albumine (BSA).

Enzyme-linked immunosorbent assay (ELISA)

Indirect ELISA

The hapten-Tg (10 μg/ml in PBS) was adsorbed on microtitre plates overnight at room temperature. Then the plates were washed three times with PBS buffer and incubated during 2 hours at 37°C in a PBS-2% non-fat milk solution to block non specific binding sites. After washing three times with PBS, plates were incubated with 0.01 μg Fab fragments in 100 μl PBS for 2 hours at room temperature. To remove unbound Fabs, plates were washed three times with PBS-0.1% Tween (PBS-T). A mixture of rabbit antibodies specific for human light chain antibodies (Dako A 192 and A 193), diluted at 1/1000 in PBS-2% non fat milk, was then incubated for 1 hour at room temperature. After washing three times with PBS-T, the plates were incubated for 1 hour at room temperature with a 1/10000 dilution of anti-rabbit IgG (whole molecule) antibodies conjugated with horseradish peroxidase (Sigma A 6154). After washing with PBS-T, a solution containing 100 µg of 3,3′, 5,5′-tetramethylbenzydine (Sigma T 2885), 50 ml of 100 mM sodium acetate (pH 6), 10 μl of 30% hydrogen peroxide was added and incubated for 15 min. The reaction was quenched by adding 50 μl of 2N sulphuric acid and the resulting absorbance was measured at 450–650 nm with an automated microtitre plate reader.

Competitive ELISA

These assays were carried out to demonstrate whether or not the Fabs could recognise the soluble form of the hapten. Competitions were also performed with molecules **C1** or **C2** to localise accurately the paratope of the hapten recognised by the Fab fragments. The procedure for the competitive ELISA was similar to the indirect one. In that case, Fabs were previously incubated with 10 μM of molecules **H**, **C1** or **C2** during one hour at room temperature before the incubation with the coated hapten.

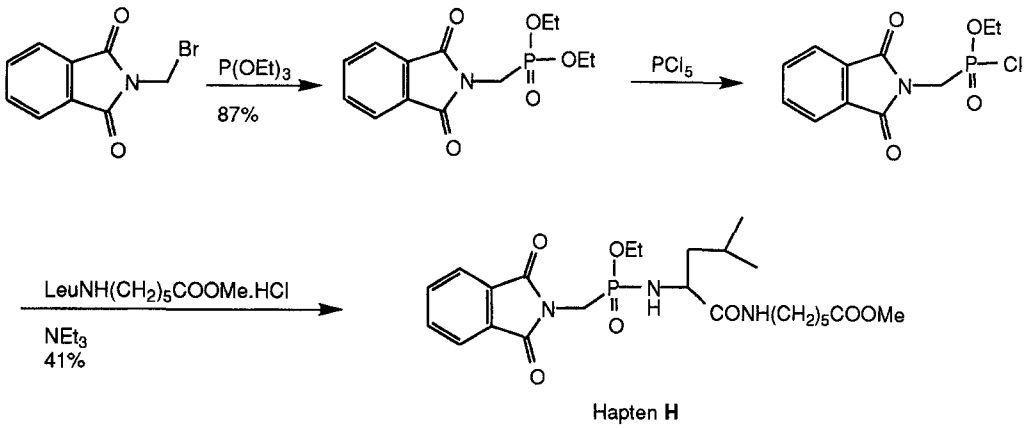
Plasmid sequencing

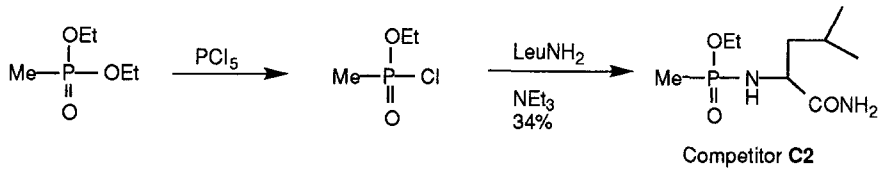
2 μg of plasmid for each of the 15 clones were used for the sequencing reaction with the following primers (Griffiths, 1994): C_κ lib seq for kappa chains, Cλ lib seq for lambda chains and pel B back for heavy chains. Sequencing reactions were performed with a T_7 sequencing kit (Pharmacia) according to the manufacturer's instructions.

Results

Hapten **H** and competitor **C2** were prepared according to a procedure adapted for Martell and al (Hariharan, 1973) as described in Scheme 2. Phosphonochloridate was generated in situ via the reaction of the corresponding phosphonate with PCl_5. Coupling reaction with a leucine derivative in the presence of NEt_3 afforded hapten **H** or competitor **C2**.

The screening of Fab fragments (Griffiths, 1994) from the "naive" library was performed by coating BSA-hapten on the surface of Immunotube (Intermed Nunc). After an incubation with the 6.5×10^{10} different Fab fragments displayed on the surface of filamentous phages, only specific phage-Fabs bound to the solid phase. A basic elution allowed us to isolate different





Scheme 2. Syntheses of hapten **H** and competitor **C2**

phage-Fabs able to recognise the hapten conjugated to the carrier protein. A cloning step allowed the expression of soluble Fab fragments in the culture supernatant. At this stage the fragments were no longer displayed on the surface of the phages. A competitive ELISA was then performed with hapten **H** and we identified 15 Fab fragments specific of the soluble form of the hapten. These 15 Fabs were sequenced, 6 of them were different. We carried out indirect ELISA test on the hapten coupled to thyroglobulin followed by a competitive ELISA test with **C1** and **C2** molecules (Fig. 1).

The 6 Fabs recognise the hapten coupled to Tg and the soluble form of the hapten **H** (results not shown). Two of them, the Fab 32B6 and 42G6 do not compete with **C1** and **C2** (there is no decrease of the optical density after incubation with the competitors) and probably bind the Pthalimide group of the hapten (Fig. 1). 32D5 and 42C8 compete with both **C1** and **C2** molecules. They probably bind the Leucine region common to the two competitors. 32D10 and 4H7 compete with **C2** but not with **C1**, thus they bind to the phosphonamidate group.

Discussion

Antibodies able to bind a transition state analogue do not often possess catalytic activity. Most, probably only those with a high affinity for the specific region mimicking the transition state of the reaction, (in our case the tetrahedral structure of the hapten **H**) can act as catalysts (Stewart, 1995).

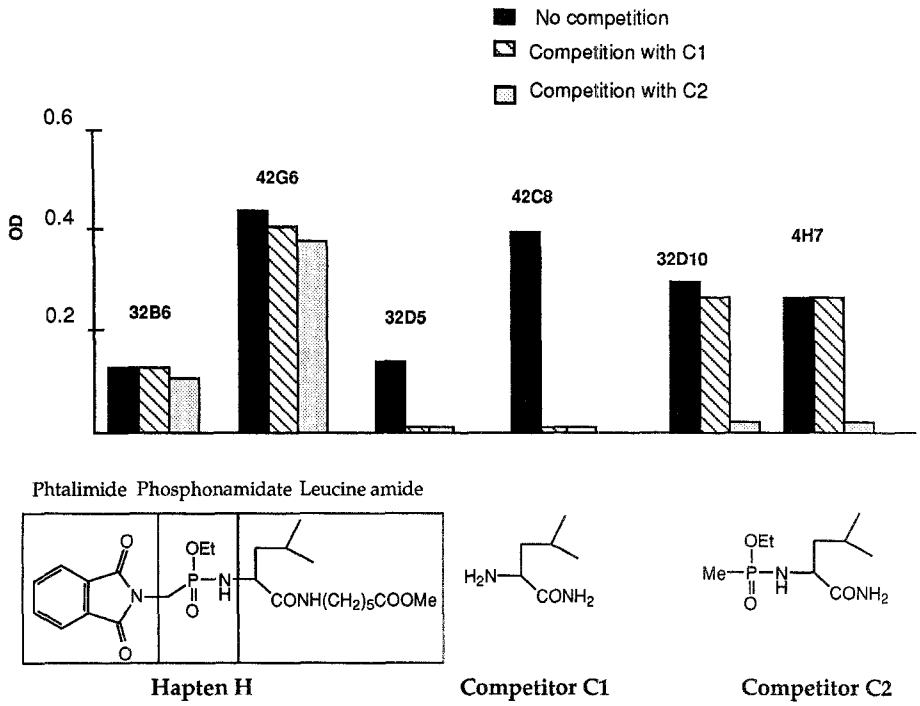


Fig. 1. Indirect Elisa with Tg-hapten and Competitive Elisa between Tg-hapten with **C1** or **C2**

Among all the Fabs we selected for binding to the hapten coupled to the carrier, only 32D10 and 4H7 recognised the phosphonamide group. Thus, these two Fabs are able to recognise accurately only one atom, namely the tetrahedral phosphorus. Unfortunately they do not present any catalytic activity in peptide synthesis in the conditions we used, perhaps due to too low affinity.

We have shown that the high complexity of the naïve library screened for binding to the hapten allowed the selection of several specific Fab fragments very quickly without any immunisation step. Thus this powerful system combined with the high specificity of recognition of the screened Fabs could be exploited for the discrimination of closely related molecules.

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