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A Mechanism-Based Probe for gp120-Hydrolyzing Antibodies

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Abstract—An antigenic peptide analogue consisting of HIV gp120 residues 421–431 (an antigen recognition site probe) with diphenyl amino(4-amidinophenyl)methanephosphonate located at the C-terminus (a catalytic site probe) was synthesized and its trypsin and antibody reactivity characteristics were studied. Antibodies to the peptide determinant recognized the peptidyl phosphonate probe. Trypsin was inhibited equipotently by the peptidyl phosphonate and its simple phosphonate counterpart devoid of the peptide determinant. The peptidyl phosphonate inhibited the gp120-hydrolyzing activity of a catalytic antibody light chain. It was bound covalently by the light chain and the binding was inhibited by the classical active-site directed inhibitor of serine proteinase, diisopropyl fluorophosphate. These results reveal that the peptidyl phosphonate ester can serve as a probe for the antigen recognition and catalytic subsites of proteolytic antibodies.

Site-specific proteolytic Abs[†] hold promise for efficient removal of microbial protein targets without indiscriminate hydrolysis of other proteins. Certain proteolytic Abs contain active sites consisting of a serine proteinase-like catalytic triad that is functionally coordinated with traditional noncovalent antigen binding forces, allowing antigen-specific stabilization of the ground state followed by nucleophilic attack on the antigenic substrate.[1] Several serine proteinase-like Abs were identified in patients with immunological disorders and animals immunized with polypeptide antigens.[2a—d] Compounds that bind such Abs selectively are essential for study of catalytic mechanism and for capture of catalysts from displayed Ab repertoires. Recently, simple phosphonate diesters have been developed as probes for nucleophiles present in displayed Ab and non-Ab serine proteinases,[3a,b] and phosphonate monoester transition state analogues have previously been employed for isolation of certain esterase Abs.[4] The mechanism-based covalently reactive compounds are the preferred probes for serine proteinase-like Abs, since they recapitulate the covalent reaction of peptide substrates with the enzyme active site.[5] The ideal mechanism-based probes, however, should contain a structural component designed to bind the Abs with the desired antigenic specificity in addition to the chemically reactive phosphonate. Here we describe the design, synthesis and Ab reactivity characteristics of a covalently reactive antigenic peptide analogue (CRA) consisting of a phosphonate diester moiety (the catalytic subsite probe) and a peptide derived from the conserved region of the HIV coat protein gp120 (the Ab recognition site probe).

Design

Diphenyl amino(4-amidinophenyl)methanephosphonate derivatives are active-site directed irreversible inhibitors of trypsin-like enzymes,[6] forming the covalent enzyme-inhibitor adducts by phosphonylating the active site Ser residue.[7] The positively charged amidino group adjacent to the phosphonate diester group serves as an analogue of Lys432 of gp120. The Lys432–Ala433 bond is the most likely specific cleavage target by Abs because (1)

[†]Abbreviations: Ab, antibody; BSA, bovine serum albumin; CRA, covalently reactive antigenic peptide analogue; DFP, diisopropyl fluorophosphate; EAR-MCA, *N-tert*-butoxycarbonyl-γ-benzyl-Glu-Ala-Arg-7-amino-4-methylcoumarin hydrochloride; HIV, human immunodeficiency virus type 1; IgG, immunoglobulin G; KLH, keyhole lympet hemocyanin; PyBOP, (benzotriazol-1-yl)oxy-tris(pyrrolidino)phosphonium hexafluorophosphate; SDS, sodium dodecylsulfate; TFE, 2,2,2-trifluoroethanol. Amino acids, peptides and their derivatives were abbreviated as recommended by the IUPAC–IUB Joint Commission on Biochemical Nomenclature: *Eur. J. Biochem.* **1984**, *138*, 9.

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most natural proteolytic Abs cleave Lys/Arg–X bonds (X represents an amino acid residue);[8a,b] (2) this bond is located in a region responsible for HIV binding by host CD4 receptors, and it's hydrolysis by trypsin is shown to induce considerable loss of the CD4-binding capability of gp120.[9] As to the peptide component, Abs can recognize peptides as short as 4–5 amino acids, but high affinity binding is usually attained with somewhat longer peptides (>10 residues).[10] Thus the undecapeptide corresponding to gp120 residues 421–431 was linked to an amino function located in the phosphonate unit (Fig. 1). The resulting molecule is intended to serve as a *message–address* system in which the gp120-derived peptide (*address*) delivers the phosphonate moiety (*message*) to the catalytic site. Biotin and Cys were introduced to the N-terminus to permit detection of the molecule and its conjugation to carrier proteins, respectively.

Synthesis

The common stepwise coupling of protected amino acids onto the phosphonate derivative was not employed to avoid potential hydrolytic decomposition of the terminal phosphonate ester moiety by successive exposure to harsh deprotection conditions. Instead, CRAs **1a–c** were synthesised by condensing the protected peptide segments (**4a–c**) and the aminoalkanephosphonate derivative as outlined in Figure 1b. Briefly, intermediate **3** was prepared by Fmoc-based solid-phase synthesis on 2-chlorotrityl resin followed by cleavage with 30% TFE–CH_2Cl_2.[11] Then biotin tags with cleavable and non-cleavable linkers and Boc-Cys(Trt) were incorporated to give **4a–c**. These were coupled with diphenyl amino (4-amidinophenyl)methanephosphonate using PyBOP. The products were deprotected with anhydrous TFA to give CRAs **1a–c**, which were purified by reversed-phase HPLC to homogeneity.[12] **1c** was conjugated with BSA using a commercially available bifunctional cross-linking agent, *N*-(γ-maleimidobutyryloxy)succinimide. Average mol/mol ratio of **1c** and BSA was 3.9.[13] Compound **2** was synthesized as described in the previous report.[14]

Ab Reactivity Characteristics

To determine the integrity of the antigenic determinant in **1a**, its binding to Abs raised to a KLH conjugate of gp120(421–436)[15] was measured by ELISA. Various dilutions (1:100–1:10000) of the antiserum from BALB/c mice immunized with gp120(421–436)–KLH were incubated in wells containing immobilized **1c**–BSA and gp120(421–436)–BSA, and Ab-binding was measured using an anti-IgG–horseradish peroxidase conjugate. The anti-gp120(421–436) Abs were bound by immobilized **1c**–BSA and gp120(421–436)–BSA with near equivalent potency (Fig. 2a). Importantly, binding of **1c**–BSA by nonimmune IgG was no greater than of gp120(421–436)–BSA, indicating that the presence of the phosphonate group does not result in nonspecific Ab phosphonylation. This result indicates that the antigenic peptide component of **1c** is indistinguishable from the peptide devoid of the phosphonate group.

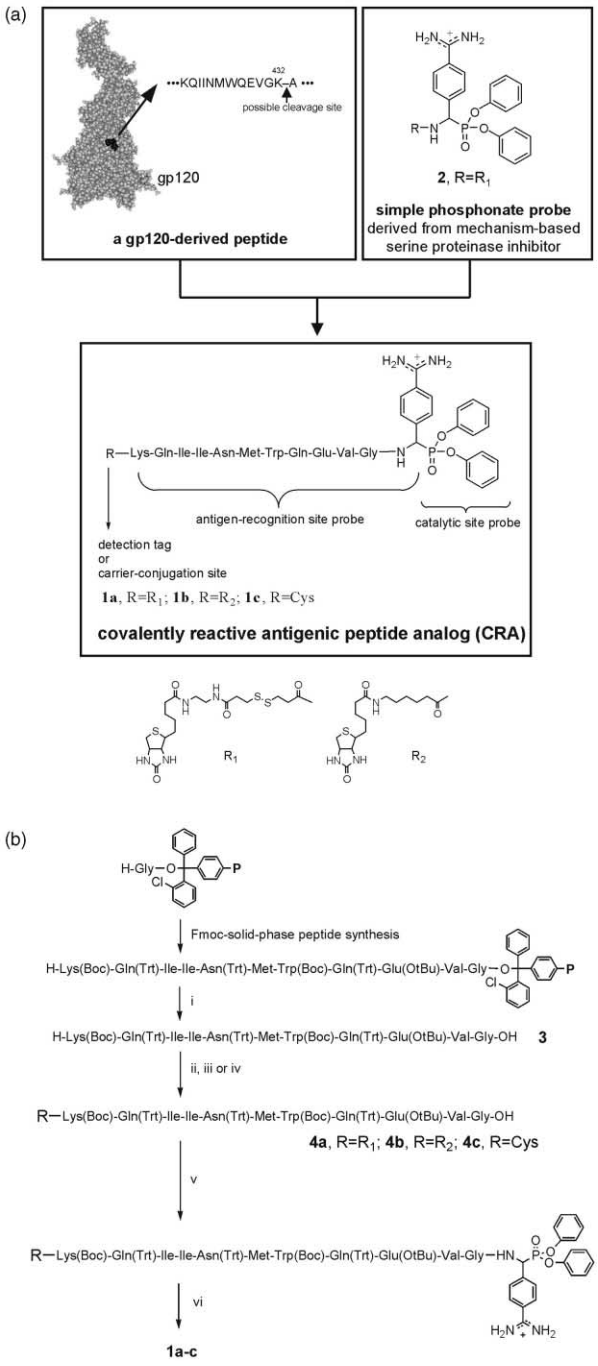

Figure 1. (a) Design of CRAs **1a–c**. CRAs consist of the peptide derived from CD4-binding region of gp120 (Ab recognition site probe) and the chemically reactive phosphonate moiety (catalytic site probe). Biotin and Cys were introduced to the N-terminus to permit detection of the molecule and its conjugation to carrier proteins, respectively. (b) Synthetic scheme for CRAs **1a–c**. Reagents and conditions: (i) TFE–CH_2Cl_2 (3:7); (ii) (2-biotinamido)ethylamido-3,3′-dithiodipropionic acid *N*-hydroxysuccinimide ester, *i*Pr_2EtN, 1-methyl-2-pyrrolidinone; (iii) (6-biotinamido)hexanoic acid *N*-hydroxysuccinimide ester, *i*Pr_2EtN, 1-methyl-2-pyrrolidinone; (iv) Boc-Cys(Trt) *N*-hydroxysuccinimide ester, *i*Pr_2EtN, 1-methyl-2-pyrrolidinone; (v) diphenyl amino(4-amidinophenyl)methanephosphonate dihydrobromide, PyBOP, *i*Pr_2EtN, DMF; (vi) TFA. **P** represents the solid support.

To assess the reactivity of **1a** with the active site nucleophiles, its interactions with two catalysts were studied: (1) the serine proteinase trypsin and (2) an Ab light chain previously identified to display gp120ase

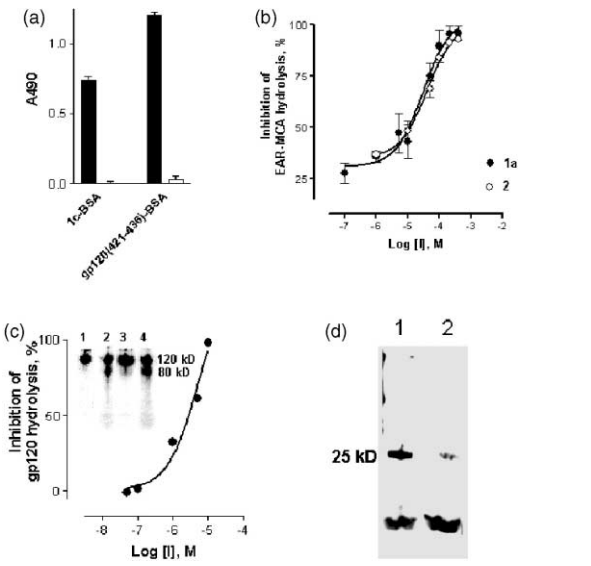

Figure 2. (a) **1c**–BSA binding by anti-gp120(421–436)–KLH Ab. Maxisorp 96-well microtiter plates were coated with BSA–gp120(421–436) and **1c**–BSA (11.2 pmol peptide equivalents/well) in 100 mM bicarbonate buffer (pH 8.6, 2 h, 37 °C). Wells were washed three times at each step with 10 mM sodium phosphate containing 137 mM NaCl, 2.7 mM KCl and 0.05% Tween-20 (pH 7.4, PBS-T) and blocked with 5% skim milk in PBS-T. Anti-gp120(421–436)–KLH serum obtained from a mouse and nonimmune mouse serum were diluted in PBS-T containing 1% skim milk (binding buffer, 1:100, 1:300, 1:1000, 1:3000, 1:10,000). Bound murine IgG was detected using peroxidase-conjugated goat anti-mouse IgG diluted in binding buffer. Histogram shown is data using 1:1000 dilution sera. (b) Inhibition of trypsin by **1a**. Trypsin (0.75 nM) was preincubated in 10 mM sodium phosphate, pH 7.4, containing 137 mM NaCl, 2.7 mM KCl, and 0.025% Tween-20 in the presence of **1a** or **2** (0.25 μM–4.0 mM, 37 °C, 30 min). EAR-MCA was added to the solutions (final concentrations: trypsin, 0.6 nM; inhibitor, 0.1 μM–0.4 mM; substrate, 0.2 mM), and amidolytic activity was determined by fluorimetric measurement of 7-amino-4-methylcoumarin generated at 470 nm with excitation at 360 nm. % Inhibition was calculated relative to fluorescence without inhibitor (616 FU/h). Background fluorescence in the absence of enzyme was 31 FU/h. (c) Inhibition of Ab light chain catalyzed ^{125}I-gp120 cleavage by **1a**. Inset, autoradiogram showing non-reducing SDS-electrophoresis gels of Ab light chain (LAY, 0.5 μM) catalyzed hydrolysis of ^{125}I-gp120 (1.1 nM, 2.3×10^3 cpm) in the presence of **1a** (10 μM; lane 3) or **2** (10 μM; lane 4). Lanes 1 and 2 show ^{125}I-gp120 incubated in assay diluent with and without the light chain (0.5 μM), respectively. 17 h, 37 °C; pretreated with inhibitor for 1 h. Plot shows inhibition of the cleavage reaction at varying **1a** concentrations calculated as the decrement of 80 kD-band intensity determined by densitometry. (d) Covalent **1a** binding by Ab light chain. Streptavidin-peroxidase stained blots of SDS-gels showing reaction mixtures of **1a** (80 μM) treated for 1 h at 37 °C with light chain LAY (1.0 μM) without (lane 1) and with DFP pretreatment (lane 2; 5.0 mM, 37 °C, 0.5 h).

activity in a random screen of monoclonal light chains from multiple myeloma patients (light chain LAY).[16] Trypsin treated with **1a** or its nonpeptidyl counterpart **2** was incubated with EAR-MCA. As shown in Figure 2b, **1a** and **2** inhibited the catalytic activity of trypsin with equivalent potency (IC_{50}: **1a**, 30 μM; **2**, 31 μM). A previous study indicates that the catalytic activity of Ab light chain LAY is inhibitable by a serine proteinase inhibitor, DFP.[16] As predicted, **1a** inhibited the light chain-catalyzed cleavage of ^{125}I-gp120, assessed from the intensity of the 80 kD product band seen by SDS-electrophoresis (Fig. 2c). At the **1a** concentration yielding near-complete inhibition of the reaction (10 μM, lane 3, Fig. 2C inset), **2** was without inhibitory effect. The covalent adduct of **1a** with the light chain was detected as a band at 25 kD by denaturing electrophoresis (Fig. 2d). Pretreatment of the light chain with DFP substantially reduced formation of the **1a**-adduct band, indicating that a serine proteinase-like catalytic site is responsible for **1a**-binding. These observations indicate uncompromised chemical reactivity of the phosphonate moiety in **1a**.

Conclusion

These results indicate that CRAs **1a**–**c** are suitable probes for Abs that combine a serine proteinase-like catalytic mechanism with the ability to recognize a conserved antigenic determinant of gp120. Such CRAs can be applied for identifying gp120-hydrolyzing Abs as stable covalent adducts from autoimmune Ab repertoires, which are known to contain gp120 specificities.[17] Furthermore, CRA–protein conjugates such as **1c**–BSA reported here are potential immunogens to induce synthesis of proteolytic Abs specific for gp120.

Acknowledgements

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12. **1a**: t_R 19.27 min (YMC-ODS AM 4.6×250 mm, 0.05% TFA aq/0.05% TFA in MeCN 80:20 to 20:80 in 40 min, 1.0 mL/min), *m*/*z* (ESI) 2171.1 (MH^+). **1b** t_R 21.78 min (the same conditions as **1a**), *m*/*z* (ESI) 2048.5 (MH^+), 2070.5 (MNa^+). **1c** t_R 18.51 min (the same conditions as **1a**), *m*/*z* (ESI) 1812.9 (MH^+).
13. **1c**/BSA ratio was calculated from reacted SH (determined by Ellman's method) and BSA concentration (determined by Bradford's method).
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