Molecular Cloning of a Proteolytic Antibody Light Chain*

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Qing-Sheng Gao†, Mei Sun‡, Sonia Tyutyulkova, David Webster§, Anthony Rees¶, Alfonso Tramontano¶, Richard J. Massey¶, and Sudhir Paul∗

From the Department of Anesthesiology and the Eppley Cancer Research Institute, University of Nebraska Medical Center, Omaha, Nebraska 68198-6830, the ‡Department of Biochemistry, University of Bath, Calverton Down, Bath BA2 7AY, United Kingdom, and ¶IGEN Inc., Rockville, Maryland 20852

The cDNA for an antibody light chain raised by immunization against vasoactive intestinal peptide (VIP) was cloned in a bacterial expression vector, and the recombinant light chain was purified to electrophoretic homogeneity. The light chain catalyzed the hydrolysis of VIP efficiently owing to its comparatively high affinity for the substrate. In control experiments, the catalytic activity was preserved at a constant level after further chromatography of the light chain on anion-exchange and gel-filtration fast protein liquid chromatography columns, and it was removed by immunoadsorption with immobilized anti-mouse light chain antibody. The amide bond linking methylcoumarinamide (MCA) and arginine in a tripeptide unrelated in sequence to VIP was cleaved by the light chain with lower affinity and kinetic efficiency (Km/Kcat). Hydrolysis of the peptidyl-MCA conjugate was inhibited competitively by the alternate substrate, VIP. The K and K values for VIP were in the same range, indicating that peptide-MCA and VIP hydrolysis occurs at a common catalytic site in the light chain. Molecular modeling suggested the presence of a serine protease-like site in the light chain. This was supported by inhibition of the hydrolytic activity by serine protease inhibitors, but not by inhibitors of other classes of proteases. These observations suggest a poorly discriminatory catalytic site, with specificity for VIP arising chiefly by means of the antigen recognition function of the light chain combining site.

Efficient catalysis by autoantibodies to vasoactive intestinal polypeptide (VIP)† (1) and DNA (2) has been reported, but the genesis of these activities and the identity of antigens inciting formation of the antibody catalysts are not clear. Since transition state lifetimes are very short, an antitransition state specificity is an unlikely explanation for the catalytic activity of autoantibodies. In analogy with the evolution of enzymes, sequence diversification occurring in antibody variable region genes (3) over the course of the immune response could result in the formation of catalytic active sites. The key test of this hypothesis is that immunization with natural antigens, as opposed to commonly used transition state mimics (4), should lead to development of catalytic antibodies. A monoclonal antibody raised to VIP displays a peptideolytic activity, but this activity is expressed only in very dilute solutions (see Ref. 5 for discussion). Consequently, this antibody is unsuitable for detailed study of catalysis and demonstration of turnover, a defining feature of a true catalyst. Here, we report efficient catalysis by the recombinant light chain subunit of the anti-VIP antibody. The activity shows preference for VIP at the substrate binding step, but turnover rates for VIP and a nonhomologous protease substrate are nearly equivalent. The presence of a serine protease-like catalytic triad in the light chain was suggested by molecular modeling and supported by inhibition of the activity by serine protease inhibitors. These observations indicate a poorly discriminatory catalytic site in the light chain, with specificity for VIP arising chiefly by means of the high affinity antigen binding function.

MATERIALS AND METHODS

cDNA Amplification and Cloning—A hybridoma cell line (clone c23.5) secreting a monoclonal antibody was developed from a mouse hyperimmunized with a VIP-keyhole limpet hemocyanin conjugate (5). cDNA preparation was by the reverse transcriptase-polymerase chain reaction method from RNA isolated from 3 x 10⁵ c23.5 hybridoma cells (6) using forward (GAGTCATTCTGCGGCCGCCTCATTCCTGTGGAGCTCTTGTTG) and reverse (GTCCTCGCAACTGCGGCCAGCGCGGATGGGCGGAYGTNGTNATGACNGAC) primers corresponding to constant region residues 206–213 and framework 1 residues 1–8 (6). Sequencing was by the dideoxy chain termination method (30) using vector-specific primers located in the vector on the 5'-side (CAGGAAACAGCTATGAC) and 3'-side (GAACTCCTCCTGCGG) of the insert. Transformation of Escherichia coli (HB2151) was by electroperoration; recombinants were grown in ampicillin; expression was induced with 1 mM isopropyl-1-thio-
β-p-galactopyranoside (2.5 h, 30 °C); and a periplasmic extract was prepared in 10 mM sodium phosphate, 1 mM EDTA, pH 7.2, 1 mM sodium chloride and dialyzed against 50 mM Tris-HCl, pH 7.4, 0.025% Tween 20.

L-chain Purification—The his tag permitted purification of the recombinant protein by two cycles of chromatography on chelating fast-flow Sepharose (Pharmacia Biotech Inc.) charged with 0.2 M nickel sulfate and equilibrated with 50 mM Tris-HCl, pH 7.4, 0.035% Tween 20 (equilibration buffer). The column was washed (2 ml/min) successively with equilibration buffer and with 2 mM ammonium chloride in equilibration buffer, 50 mM sodium formate, pH 3.8, 0.025% Tween 20 and eluted with 50 mM EDTA in equilibration buffer. Light chain detection was by dot blots for the C-terminal c-myc tag using samples (50 μl) plated on 0.2-μm nitrocellulose (BA83, Schleicher & Schuell) in a Bio-Rad blotting apparatus, blocked (2 h) with 5% bovine serum albumin in phosphate-buffered saline, treated (60 min each) with mouse anti-c-myc antibody (1:50 dilution of ascites; clone 9E10, American Type Culture Collection) and goat anti-mouse IgG conjugated to horseradish peroxidase (1:1000 dilution; Sigma), and stained with 0.03% (w/v) dimino- benzidine (Sigma) in 0.03% H₂O₂. EDTA fractions containing c-myc-
stainable light chain were dialyzed and rechromatographed on light chain. Shown on the IEF gel are silver-stained dication was dialyzed against assay diluent prior to determination of hydrolytic "is-HCl, pH 7.4.0.025% sodium azide with the following antibodies immobilized on Sepharose 1,000 columns (Pharmacia Biotech Inc.; 0-1 M NaCl) and from the Mono-Q column (elution position at 0.5 M Tris-HCl, pH 7.7, 0.025% Tween 20). Light chain expression levels were 1-2 mg/liter of bacterial culture, and the yield of the pure protein was 350 μg/liter. Isoelectric focusing was on Phast IEF gels, pH 3-9, and SDS-polyacrylamide gel electrophoresis was on 8-25% Phast acrylamide gels. SDS gels were diffusion-blotted onto nitrocellulose membranes for 30 min and stained with anti-c-myc antibody. The N-terminal amino acid sequence of the recombinant light chain (2.5 μg) adsorbed on a polyvinylidene difluoride membrane (ProSpin cartridge, Applied Biosystems Inc.) was determined by automated Edman degradation as described (7). Anion-exchange chromatography was on a Mono-Q column (Pharmacia Biotech Inc.; 0-1 M NaCl (30 min) in 50 mM Tris-HCl, pH 7.4, 0.025% Tween 20), and gel-filtration chromatography was on a Superose 12 column (Pharmacia Biotech Inc.; 0.5 ml/min) (10). Light chain recovered from the gel filtration column (retention time of 28 min) and from the Mono-Q column (elution position at 0.43 M NaCl) was dialyzed against assay diluent prior to determination of hydrolytic activity. Immunoadsorption of the light chain (7.5 μg) was for 17 h (4 °C) in 50 mM Tris-HCl, 100 mM glycerine, pH 7.7, 0.025% Tween 20, and 0.02% sodium azide with the following antibodies immobilized on Sepharose (3 ml of settled gel): mouse anti-c-myc (clone 9E10), rat anti-mouse κ-chain (Zymed Laboratories, Inc.), and rat anti-mouse IgGl (Zymed Laboratories, Inc.). Preparation of immobilized anti-c-myc and the immunoadsorption method were described (11). The light chains from reduced and alkylated antibody ω23.5 and a control nonimmune antibody from myeloma IgGκ,κ; UPC10, Sigma) were purified in 6 x guanidine hydrochloride and renatured by dialysis as described (7).

VIP Hydrolysis—Synthetic VIP (HSDAVFTDNYTRLKKQMAVKKY LNSILN-NH₂, peptide content of 81%; Bachem California) was labeled with ¹²⁵I using chloramine T, and [¹²⁵I-Tyr¹⁰]VIP was separated by reversed-phase HPLC and identified by N-terminal radiosequencing (1, 12). [¹²⁵I-Tyr¹⁰]VIP was treated with the L-chain at 38 °C in 200 μl of 0.05 M Tris-HCl, 0.1 M glycerine, pH 7.7, 0.025% Tween 20, 0.1% bovine serum albumin, and peptide hydrolysis was estimated by measuring the radioactivity soluble in 10% trichloroacetic acid (1). Estimates of peptide breakdown by this method are essentially identical to those obtained by reversed-phase HPLC separation of intact and degraded peptide fragments (7). Trypsin from bovine pancreas (3 x crystallized, 3080 units/mg) was from United States Biochemical Corp. Disisopropyl fluorophosphate, aprotinin, iodoacetamide, and peptatin A were from Sigma.

Peptide MCA Hydrolysis—Peptide-MCA conjugates (Peptides International Inc.) were treated with the catalyst in 96-well microplates (Microfluor W, Dynatech Laboratories Inc.) in 125 μl of 0.05 M Tris-HCl, 0.1 M glycerine, pH 7.7, 0.025% Tween 20 (6 h, 37 °C; λ₃₇₀ = 370 nm and λ₅₄₀ = 460 nm; Perkin-Elmer LS-50 spectrofluorometer equipped with a plate reader).

Molecular Modeling—A model of the V₉₂/V₉₈ complex was prepared using AbM™ (Oxford Molecular, Inc.) (13, 14) and minimized by a combination of steepest descent and conjugate gradient methods using DISCOVER (Biosym Technologies). Greater than 98% of the amino acids of the model fell in the allowed regions of a Ramachandran plot. The protocol initially fixed all heavy atoms, followed by gradual relaxation of tethering constraints on the side chains and then relaxation of constraints on the backbone atoms of the CDRs. A mirror image of the catalytic triad of subtilisin (Asp³⁴, His⁶⁴, and Ser⁴⁵) (15) was fitted to light chain residues Asp¹, Ser¹⁴, and His⁸⁰ by superposition of their Ca atoms. Terminal atoms of the antibody side chains were template-forced.

![Natural Catalytic Antibody Light Chain](image)
to their catalytic counterparts and allowed to relax with no constraints on the system. N-terminal Asp was not well aligned with Asp of subtilisin, but when all 3 light chain residues were template-forced to the catalytic triad of the enzyme followed by relaxation of the structure, good superposition was evident. The manipulations did not unduly perturb the light chain backbone conformation, and the conformations of the canonical CDRs (L1 and L3) were retained (16).

RESULTS AND DISCUSSION

Catalytic Activity of the Light Chain—Polymerase chain reaction-amplified light chain cDNA was inserted into a bacterial expression vector, and the recombinant protein was purified by means of its affinity for metals (Fig. 1). The purified light chain preparations contained a single protein migrating within the VIP-binding site, and the N terminus was derivatized with an c-myc tag (the recombinant protein contains a 10-residue c-myc tag). N-terminal sequencing of the purified protein yielded a single amino acid sequence (Asp-Val-Val-Met-Thr) corresponding to the previously reported sequence of the light chain purified from reduced and alkylated light chain renatured on assumption (16).

| Catalyst | Substrate | $k_v$ | $k_{cat}$ | $k_{cat}/K_v$ |
|----------|-----------|-------|-----------|---------------|
| L-chain  | VIP       | 0.2  | $10^{-4}$ | 1.1 $10^{-2}$ |
| L-chain  | Pro-Phe-Arg-MCA | 11.5 | $10^{-4}$ | 6.8 $10^{-3}$ |
| Trypsin  | VIP       | 3.8  | $10^{-4}$ | 1.1 $10^{-3}$ |
| Trypsin  | Pro-Phe-Arg-MCA | 2.0  | $10^{-4}$ | 2.6 $10^{-3}$ |

Table I

Hydrolysis of peptide-MCA substrates by the anti-VIP light chain

Table II

Catalytic Activity of the Light Chain—Polymerase chain reaction-amplified light chain cDNA was inserted into a bacterial expression vector, and the recombinant protein was purified by means of its affinity for metals (Fig. 1). The purified light chain preparations contained a single protein migrating within the VIP-binding site, and the N terminus was derivatized with a benzoyl group. The N terminus was derivatized with a t-butyloxycarbonyl group.

2 Reversed-phase HPLC was as described (10). Peptide hydrolysis was estimated based on the decrease in area of the intact VIP peak eluted at 46% acetonitrile. Product identities are to be reported elsewhere.

3 The N terminus was derivatized with a benzoyl group.

4 The N terminus was derivatized with a t-butyloxycarbonyl group.
**FIG. 2.** Inhibition of light chain-catalyzed hydrolysis of Pro-Phe-Arg-MCA by VIP. Reaction rates were measured at 1 µM light chain and 25 (●), 15 (▲), or 7.5 (●) µM substrate. Other conditions were as described in the legend to Table I.

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$\frac{1}{rate}, \text{mM/hour}^{-1}$

$[-K_i, \mu M]$  

1 GAT GTG GTG ATG ACA GAG ACT CTA CCT TTA GGT GTT ACC ATT asp val val met thr gln pro leu thr pro leu thr ile 1

46 GCA CCA GCC TCC TCT TGC TGC AAC TGA AGG CAG ACC CTC TTA gln pro ala ser ile ser lys ser ser gln ser leu leu 14

91 CAT ACT GAT GGA AGG ACA TAT TGC ATT TGC TGA TTA CAG ACG his thr asp gly lys thr thr leu leu leu leu 18

136 GGG GAC CTA TTC TCT TGC CTA AAA CAA GAC gly ser pro pro pro pro leu ile tyr leu val ser lys leu asp 46

181 TGC GTC GCC AGG TGC AAG AAC TTA AAA GAG AAG gly ser pro ser pro pro pro pro pro pro pro pro ser 61

226 TTC ACA CTG AAA ATC AGC AGA GTC GAG GCT GAT GAT GAT GAG TCT GTA GAT phe thr leu ile ile ser arg val glu ala glu asp leu gly val 76

271 TAT TAT TGC CAA GGT GAT ACA CAT TCT CAG ACA TTC GGT GGA tyr tyr tyr cys trp gln gln thr his phe pro gln thr thr phe gly 91

316 GCC ACC AGG CTG GAA ATC AAA gly thr leu glu ile leu 106

**FIG. 3.** Nucleotide sequence and deduced amino acid sequence of the anti-VIP light chain inserted in pCANTAB5His$\beta$g (GenBank accession number L34775). Complementarity-determining regions are underlined.

$10^3$ min$^{-1}$. A nonimmune light chain (from myeloma IgG, K; UPC10, Sigma) purified and renatured under identical conditions was devoid of catalytic activity.

**Molecular Model of the Light Chain**—Inspection of the nucleotide sequence and the deduced amino acid sequence of the $\lambda$I region (Fig. 3) showed it to be a member of $\kappa$-chain family II (19). A $\lambda$I-$\gamma$ dimer model of antibody c23.5 was produced, the $\lambda$I domain of which is shown in Fig. 4. This model is relevant to the properties of the light chain since dilute solutions of monoclonal c23.5 IgG preparations express a weak peptidolytic activity (5); the recombinant single chain $\lambda$I-$\gamma$ construct (Fv) of this antibody also expresses catalytic activity; and the structure of $\lambda$I domains is often largely independent of the mode of

3 S. Paul and Q.-S. Gao, unpublished data.

**FIG. 4.** Rendered images of the anti-VIP $\lambda$I region after template forcing and relaxation of the structure. A shows a backbone trace of the light chain region of the modeled $\lambda$I region before (green) and after (white) template forcing and relaxation. Where the backbones did not differ, a single color with a reduced thickness for the backbone is shown. B illustrates the similar geometry of light chain residues Asp$\beta$, Ser$\beta$, and His$\beta$ (white) to the catalytic triad (red) of subtilisin after template forcing of the structure. C is a rendered image of the $\lambda$I region angled at ~30° to the plane of the combining site, showing the Asp$\beta$ (red), Ser$\beta$ (green), and His$\beta$ (blue) triad. A–C were produced using MOLSCRIPT (27), RASTER3D (28), and GRASP (29), respectively. The nucleotide sequence of the $\lambda$I region (clone c23.5) has been deposited in GenBank (accession number pending).

association with $\gamma$ domains, even where the intersubunit contacts are not conserved between the light and heavy domains (20). The model revealed two potential catalytic sites with a composition similar to that found in the catalytic triad of many serine proteases (an Asp, a Ser, and a His located in a hydrophobic pocket). The first site (Fig. 4B) contains the N-terminal aspartic acid residue of the light chain (Asp$\beta$), a serine from
CDR1 (Ser^{28}), and a histidine from CDR3 (His^{38}). The Cz distance geometries and side chain orientations of these residues were similar to those of their counterparts from the catalytic triad of subtilisin (15). The second site involved residues from CDR1 (Ser^{28}, His^{31}, and Asp^{35}). Template forcing of this site to the catalytic geometry of subtilisin disrupted the canonical CDR1 geometries and side chain orientations of these residues.

Inhibitors of other classes of proteases, including iodoacetamide (2 ms), EDTA (2 ms), and pepstatin A (500 μm), were essentially without effect on the activity. These observations suggest that where efficient catalysis of energetically difficult reactions by antibodies is seen, known enzymatic mechanisms that have evolved over many millions of years will be reproduced.

Conclusion—Polyclonal autoantibody light chains display catalytic activity (21). Some light chains secreted by B-lymphocyte tumors express sequence similarities to serine proteases (22). Light chains contribute a significant proportion of the antibody-antigen contact surface (23), and they can independently bind antigens (7, 24). The light chain described here was raised by immunization with the substrate (VIP), indicating that synthesis of catalytic sites is an intrinsic component of the immune response to polypeptide antigens. The distinctive property of the light chain not shared by proteolytic enzymes is an ability to strongly recognize the ground state of VIP. The lower Ka of the light chain for VIP compared with the nonhomologous substrate is consistent with the clonal selection theory, i.e. the light chain has been selected during the immune response to VIP because it recognizes the inciting antigen with comparatively high affinity. Polypeptide binding by antibodies can involve contacts at >15 residues in each molecule (23), but most of the binding energy appears to derive from a subset of these contacts (25, 26). In the case of a catalyst, such a multiplicity of contacts makes it possible that distinct subsites play important roles in the binding and hydrolyzing functions. This is supported by hydrolysis of Pro-Phe-Arg-MCA by the anti-VIP light chain, a substrate unrelated in sequence to VIP. The turnover rate for VIP is no higher than for the nonhomologous substrate, pointing to the possibility of an independent evolution of the hydrolytic and antigen binding functions. Even so, it is clear that the hydrolytic function is preserved in the affinity-matured light chain. Therefore, the immune system should provide a rich source of catalysts that combine a hydrolytic function with strong substrate binding affinity.

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REFERENCES

1. Paul, S., Valle, D. J., Beach, C. M., Johnson, D. R., Powell, M. J. & Massey, R. J. (1989) Science 244, 1158–1162
2. Shuster, A. M., Gololobov, G. V., Kovalusk, O. A., Bogomolova, A. E., Smirnov, V. G. & Gashkov, A. G. (1992) Science 256, 665–667
3. Tonegawa, S. (1983) Nature 302, 575–581
4. Tramontano, A., Janda, K. D. & Lerner, R. A. (1986) Science 235, 1558–1573
5. Paul, S., Sun, M., Mody, R., Tewary, H. K., Mehrta, S., Gianferrara, T., Meldal, M. & Tramontano, A. (1991) J. Biol. Chem. 267, 13142–13145
6. Chomczynski, P. & Sacchi, N. (1987) Anal. Biochem. 162, 156–159
7. Sun, M., Li, L., Gao, Q.-S. & Paul, S. (1994) J. Biol. Chem. 269, 734–738
8. Hoogenboom, H. R., Griffiths, A. D., Johnson, K. S., Chiswell, D. J., Hudson, P. & Winter, G. (1989) Nucleic Acids Res. 19, 4133–4137
9. McCafferty, J., Fitzgerald, K. J., Earnshaw, J., Chiswell, D. J., Link, J. Smith, R. & Riech, J. (1994) Appl. Biochem. Biotechnol. 47, 157–173
10. Paul, S., Sun, M., Mody, B., Eklund, S. H., Beach, C. M., Massey, R. J. & Hamel, F. (1991) J. Biol. Chem. 266, 16218–16234
11. Paul, S., Valles, D. J. & Sun, M. (1990) J. Immunol. 145, 1196–1199
12. Mody, R. K., Tramontano, A. & Paul, S. (1994) Int. J. Pept. Protein Res. 44, 441–447
13. Martin, A. C. R., Cheetham, J. C. & Rees, A. R. (1991) Methods Enzymol. 203, 121–155
14. Pedersen, J., Searle, S., Henry, A. & Rees, A. R. (1992) ImmunoMethods 1, 126–136
15. Bode, W., Papamokos, E. & Musil, D. (1987) Eur. J. Biochem. 166, 673–692
16. Chothia, C. & Lesk, A. M. (1987) J. Mol. Biol. 196, 901–917
17. Sarath, G., De La Motte, R. S. & Wagner, F. W. (1989) in Peptidyl Peptidases, ed. by T. M. Wilson, pp. 151–157, Springer, Berlin
18. Sun, M., Li, L., Gao, Q.-S. & Paul, S. (1995) Proc. Natl. Acad. Sci. USA 92, 739–753
19. Mody, B., Eklund, S. H. & Paul, S. (1991) J. Biol. Chem. 266, 15571–15574
20. Kもし, S. & Greller, L. D. (1974) Nature 251, 253–255
21. Davies, D. R., Padlan, E. A. & Sheriff, S. (1979) Annu. Rev. Biochem. 49, 379–414
22. Mahanna, W., Jacqueur, F. & Ermesov, M. (1994) Scand. J. Immunol. 39, 187–190
23. Nozoty, J., Brucodori, R. E. & Salo, P. A. (1989) Biochemistry 33, 4735–4749
24. Kelley, R. F. & O'Connell, M. P. (1993) Biochemistry 32, 6826–6835
25. Krasil, P. J. (1991) J. Appl. Crystallogr. 24, 971–990
26. Bacon, D. J. & Anderson, W. F. (1988) J. Mol. Biol. 6, 219–220
27. Nicholls, A., Bharadwaj, R. & Honig, B. (1995) Biophys. J. 64, 416
28. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467