Vol. 204, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 14, 1994 Pages 57-62

AMIDASE ACTIVITY OF HUMAN BENCE JONES PROTEINS

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Received August 23, 1994

Summary: Bence Jones proteins purified from urine of patients with multiple myeloma were found to be capable of hydrolyzing carbobenzoxy-L-valyl-glycyl-L-arginine *p*-nitroanilide (Chromozym TRY) and benzoyl-L-arginine *p*-nitroanilide (BApNA), synthetic chromogenic substrates for trypsin. The amidolytic activity obeyed classic Michaelis-Menten kinetics, exhibiting optimal activity around pH 8.4 and apparent Km of 140-730 μM and 18-27 μM for Chromozym TRY and BApNA, respectively. No activity was detected with intact IgG or Fab fragment, whereas the activity comparable to those of Bence Jones proteins was found with light chain derived from inactive IgG. Several lines of circumstantial evidence indicate that the observed activity was not due to contaminating enzyme. © 1994 Academic Press, Inc.

During the course of studies on the proteinase inhibitors in plasma and urine (1, 2), we applied the Uriel-Berges method (3) to locate the inhibitory activity on the electrophoretogram of normal human urinary proteins. This method consists of (i) gel electrophoresis of a sample presumed to contain inhibitory activity, (ii) incubation of the gel with proteinase to allow the formation of enzyme-inhibitor complex, and (iii) detection of enzyme activity by transferring the gel to chromogenic substrate solution. The whole surface of the gel is stained except for the area where the inhibitor is present. Normal urine showed a 25-kDa band stained yellowish with a tint somewhat different from either active or inactive protein. In order to identify the protein with this unusual staining property, the band was cut out of the electrophoresis sheet, and its N-terminal amino acid sequence was analyzed. The result showed that it was a mixture of immunoglobulin light chains. To get further insight into this observation, monoclonal Bence Jones proteins (BJPs) were isolated from urine of patients with multiple myeloma, and their mode of interaction with proteinase substrate was studied. To our surprise, all BJPs examined (four of κ type and one of λ type) were found to be capable of hydrolyzing carbobenzoxy-L-valyl-glycyl-L-arginine *p*-nitroanilide (Chromozym TRY) and benzoyl-L-arginine *p*-nitroanilide (BApNA), synthetic chromogenic substrates for trypsin. The present paper describes this unexpected results.

Materials and Methods

Bence Jones proteins were purified to homogeneity from urine of patients with multiple myeloma by three successive column chromatographies on Butyl Cellulofine, concanavalin A-

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Sepharose, and Sephadex G-75 followed by preparative electrophoresis (4). The final preparations were homogeneous by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under nonreducing conditions. By comparing the migration of the sample with those of the standard proteins as described (1), a molecular mass of about 25 kDa was obtained. These proteins immunoreacted with either anti-κ or anti-λ antibody, but not with anti-heavy chains (γ, μ, or α). Human IgG1, IgG2, IgG3, IgG4, IgM, and IgA purified from respective myeloma patients as well as rabbit antibodies raised against heavy (H) and light (L) chains of human IgG were obtained from Medical & Biological Labs Co Ltd. L chain was prepared from IgG1 (1 mg/0.3 ml) by reduction with 50 mM dithiothreitol for 1 h at room temperature, followed by column chromatography on protein A conjugated to Sepharose (2 ml gel, Pharmacia) to remove H chain. The flow-through fraction contained L chain. Fab was prepared from IgG1 by treatment with immobilized papain followed by chromatography on protein A-Sepharose (5). Anti-κ antibody was complexed with protein A-Sepharose as follows; anti-κ antiserum (1 ml) was applied to a column (1.6 x 1.0 cm) of protein A-Sepharose which had been equilibrated with 1.5 M glycine-NaOH buffer, pH 8.9, containing 3 M NaCl, and washed with the same solvent to remove unabsorbed proteins. Chromozym TRY and BApNA were purchased from Boehringer and Protein Research Foundation (Japan), respectively. Amidolytic activity was measured using Chromozym TRY or BApNA as substrate as described previously (6).

Results and Discussion

Figure 1 shows the N-terminal amino acid sequence of a purified monoclonal BJP, here tentatively referred to as BJP (HIR), in comparison with that of $V_{\kappa II}$ (REI), variable region of an L chain belonging to subgroup II of κ type (7), which showed the highest sequence homology among the proteins listed in the SWISS-PROT database (release 27). Since spontaneous hydrolysis of Chromozym TRY was observed at high pH, the rate of amidolysis was determined as a function of pH (Fig. 2A). Addition of bovine serum albumin at the same molar concentration as BJP (HIR) caused no stimulation of hydrolysis, indicating that the stimulation of amidolysis by BJP (HIR) is not a nonspecific action of protein. Virtually no hydrolysis was observed in the control assay at pH below 6.5, while in the presence of BJP (HIR) unambiguous activity was seen at pH range of 4.0 to 9.4. The highest activity was observed around pH 8.4. When BJP(HIR) (1 mg/ml) was applied to a column (1.6 x 1 cm) of anti-κ antibody bound to protein A-Sepharose, no activity was detected in the flow-through fraction or washing which was most likely to contain contaminant enzymes. In contrast, the fraction eluted from the column with 0.1 M sodium citrate buffer, pH 4.0, had the activity amounting to 90% of the original sample. These results support the interpretation that the amidase activity was not due to contaminating enzyme(s). The activity was not inhibited by 1 mM EDTA, eliminating the possibility that it was due to the presence of contaminating metalloproteinase or trace amount of metal ion, possible nonspecific catalyst. In order to exclude the spontaneous hydrolysis, kinetic parameters were determined at pH 6.5. The initial rates of hydrolysis was proportional to time and concentration of BJP (HIR) (Fig. 3A). The rate measured as a function of substrate concentrations followed Michaelis-Menten kinetics (Fig. 3B). By assuming that one

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                          10        20        30        40
κ(HIR)DIQMTQSPSSLSASIGDYVTITCQARQAIIDYLNWYQQWPGRAPWCLIY
κ(REI)--------------V--R-------S-D--K-------T--K--KL---
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Fig. 1. N-Terminal amino acid sequence of BJP(HIR) in comparison with $V_{\kappa II}$ (REI). Dashes represent identical amino acid residues.

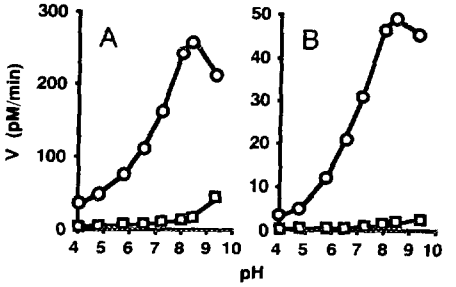

Fig. 2. Hydrolysis of Chromozym TRY (A) and BApNA (B) by BJP as a function of pH. Reaction mixtures contained 0.1 M citrate buffer (pH 4.0 and 4.8), 0.1 M phosphate buffer (pH 5.8 and 6.5) or 0.1 M Tris-HCl buffer (pH 7.2 to 9.4) with 1.55 mM Chromozym TRY (A) or 1.0 mM BApNA (B) in a total volume of 1 ml. The activity was assayed as described previously (6) in the presence (open circle) or absence (open square) of 0.4 mM (as monomer) BJP(HIR). Virtually no effect was observed by the addition of bovine serum albumin (0.4 mM), and the data are not shown.

molecule of L chain contains one active site, the rate of hydrolysis in the presence of BJP (HIR), k_{cat}, was determined to be 0.08 min^{-1}, at 36°C. At the same pH which is not optimal for enzymic activity, trypsin showed an apparent k_{cat} of 7.2 x 10^5 min^{-1}, implying that trypsin is about 10^7 times more active than BJP(HIR). Since the fact that Chromozym TRY is relatively unstable in aqueous solution may obscure the result, hydrolysis of BApNA, more stable substrate, was also studied. The results were essentially the same as those obtained for Chromozym TRY (Fig. 2B). Furthermore, three other BJPs exhibited similar amidolytic activity, kinetic parameters of which are listed in Table I. Under the same conditions, BJP(HIR) showed the activity towards H-D-Val-Leu-Arg-*p*NA (S-2266, substrate for pancreatic kallikrein) and Z-Ile-Glu-Gly-Arg-*p*NA (S-2222, substrate for coagulation factor Xa), but not towards H-D-Pro-Phe-Arg-*p*NA (S-2302, substrate for plasma kallikrein), H-D-Val-Leu-Lys-*p*NA (S-2251, substrate for plasmin), or Suc-Ala-Ala-Ala-*p*NA (substrate for elastase). The results indicate that BPJ(HIR) has a rather narrow specificity towards the arginine residue adjacent to small-sized neutral amino acids. Under the same conditions, virtually no activity was detected with immunoglobulins G1, G2, G3, G4, M, A or Fab fragment prepared from IgG1. However, L chain prepared from inactive IgG1 showed activity comparable with those of the above BJPs (data not shown). These results also argue against the interpretation that the observed activity was due to simple contamination of enzyme, and are consonant with the previous finding of naturally occurring catalytic autoantibodies to human vasoactive intestinal peptide: the hydrolytic activity of L chain of an autoantibody was 35-fold greater than that of the intact IgG or its Fab (8). The present finding that apparent Km values of BJP for BApNA were about 5-fold smaller than that of trypsin (Table I) is also consistent with the previous results that some randomly selected Ig L chains have high affinity to small aryl compounds such as nitrophenol (9). It remains to be determined, however, whether this property is related to the 'pseudoenzymic' activity of BJPs observed above.

The amidolytic activity of BJP(HIR) was inhibited by 0.1 mM (*p*-amidinophenyl)methanesulfonyl fluoride, inhibitor for serine proteinase, but not by 10 μM E-64 (epoxysuccinyl leucyl

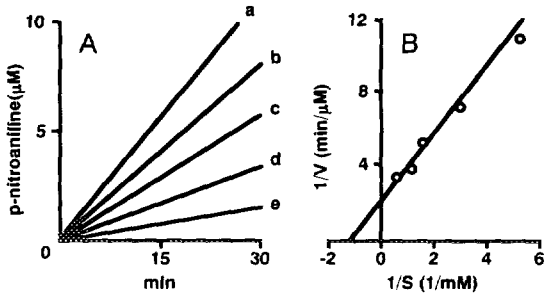

Fig. 3. Kinetic analysis of hydrolysis of Chromozym TRY by BJP(HIR). (A) Time course of the reaction. The assay was carried as described in the legend to Fig. 2 except that the pH was fixed at 6.5. Curves *a–e* represent the results obtained with BJP concentrations of 400, 320, 240, 160, and 80 μM (final concentration as monomer), respectively. (B) Lineweaver–Burk plot of the data. Each point represents the value obtained with 0.18, 0.36, 0.54, 0.70 and 1.12 mM Chromozym TRY.

agmatine), inhibitor for cysteine proteinase. The activity was lost by heating at 100°C for 5 min or treatment with 6 M urea. Since these results suggest that the nature of amidolysis by BJP was similar to that of serine proteinases, putative active site serine was titrated by the method of Chase and Shaw (10). Immediately after addition of the titrant, a burst of *p*–nitrophenol (0.2 mol/mol of BJP monomer) was observed at either pH 8.2 or pH 4.0. This suggests that approximately 20% of BJP monomer contains active serine, further ruling out the possibility that the observed amidase activity was due to trace amounts of contaminating enzyme. When subjected to gel filtration on a column (1 x 30 cm) of Superose 12, BJP(HIR) eluted as a single peak of monomer at pH 4.0, or as two peaks (monomer and dimer in an approximate weight ratio of 1:4) at pH 7.5. This result is consistent with the previous findings that Ig L chains are monomer–dimer systems between pH 5 and 8 having a pH–dependent association constants of 10^4–10^5 M^{-1} (11). All three peaks from the above column had the amidase activity at pH 4.0 or pH 7.5. The reason why only 20% of the sample reacted with active site titrant is not clear at present. Certain portions of the molecule may have been denatured or may have different conformation which does not react with the titrant.

In 1974, in a paper entitled "Do immunoglobulins have proteolytic activity", Erhan and Greller (12) pointed out the statistically highly significant similarity of structure between antibody light chains and active site regions of serine proteinases. Since then, however, virtually no attention has been paid to this issue. It is known that serine proteinases contains catalytic

TABLE I Kinetic parameters for amidolysis by monoclonal BJPs and trypsin

BJP	Isotype	Chromozym TRY		BApNA	
		Km (μM)	k_{cat} (min^{-1})	Km (μM)	k_{cat} (min^{-1})
HIR	κ	730	0.08	27	0.14
MOR	κ	470	0.18	21	0.07
YUK	κ	140	0.34	19	0.15
YAM	λ	610	3.6	18	0.60
trypsin		530	7.2×10^5	110	40

device characterized by a triad composed by serine, histidine, and aspartic acid residues, and that enzymic hydrolysis consists of at least three steps; formation of Michaelis-Menten complex, acylation and deacylation of active site serine residues of enzyme. Using site-directed mutagenesis, Carter and Wells (13) prepared a series of mutant proteins of subtilisin which are incapable of proceeding by the assumed mechanism because of the replacement of triads by other amino acids. These mutants, however, were found to be active in hydrolyzing the substrate; for example, a triple mutant in which the catalytic triad had been replaced by three alanines was still capable of hydrolyzing a peptide *p*-nitroanilide at about 2800-fold enhanced rate than uncatalyzed reaction. These results suggest that the hydrolytic activity of the mutant proteins is derived from the structure that stabilizes the transition state complex in a manner different from the authentic catalytic triad. Although the exact mechanism of amidolysis by BJPs remains to be defined, they may act like conventional amidases or like the subtilisin mutants whose triad has been replaced. Recently, numerous monoclonal antibodies which can catalyze more than 50 chemical reactions have been elicited (14). In contrast to the diversity of reaction studied, little attention has been paid to the amidolysis. Janda *et al.* (15) obtained a monoclonal antibody which accelerated amidolysis of 4-glutarylaminophenylacetic acid *p*-nitroanilide about 2.5 x 10^5-fold over the uncatalyzed reaction. The Fab fragment had approximately the same activity as the intact IgG molecule, but no data were presented with its L chain. They reported, however, that several control IgGs were not catalytic, which is in agreement with the present result that intact immunoglobulins were inactive.

Further elucidation of the 'pseudoenzymic' activity of BJP will lead to better understanding not only of the molecular mechanism underlying the hydrolysis by enzyme and catalytic antibody but also of the evolution of the enzyme catalysis. Moreover, it is possible that this 'pseudoenzymic' activity of BJP may be responsible for some of diverse symptoms associated with multiple myeloma. Studies along these lines are in progress in this laboratory.

Acknowledgments

We are indebted to Prof. A. Horiuchi and members of his department, Kinki University School of Medicine, for helpful discussion and for collecting urine samples from which BJPs were prepared.

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