Altered cleavage site preference of a proteolytic antibody light chain induced by denaturation

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Abstract A recombinant antibody light chain (L chain) maintained under non-denaturing conditions displayed preferential cleavage of synthetic peptides conjugated to methylcoumarinamide (MCA) on the C-terminal side of Arg and Lys residues. The same L chain renatured from a denaturing solvent (guanidine hydrochloride) acquired the capability of cleaving Tyr–MCA and Leu–MCA bonds, and its ability to cleave MCA linked to basic residues was decreased. The altered cleavage preference was accompanied by a conformational transition in the protein, evident from the fluorescence emission spectra. These observations suggest the feasibility of redirecting the cleavage specificity via alterations in the conformation of proteolytic antibody combining sites.

Key words: Catalytic antibody; Substrate specificity; Antibody engineering

1. Introduction

The biological functions of a proteolytic compound depend, in part, upon the particular peptide bond(s) it cleaves. The fragments produced by cleavage of polypeptide substrates at different peptide bonds often express different levels of residual biological activity and, in certain cases, the fragments can express new activities not associated with the intact polypeptide [1–3]. Changes in the scissile bond specificity of a catalyst can be anticipated, therefore, to influence the biological consequences of the proteolytic reaction. The recombinant light chain (L chain) of an antibody raised to vasoactive intestinal polypeptide (VIP) contains a serine protease like catalytic triad (H, S and D residues; amino acids shown in one-letter code) [4,5]. The peptidase activity of the L chain can be monitored by measuring the cleavage of the amide bond linking methylcoumarinamide to basic residues [4]. We describe here a change in the cleavage preference of the L chain induced by denaturation with guanidine hydrochloride.

2. Materials and methods

The recombinant L chain (clone c23.5) expressed in the periplasm of a bacterial host was purified to electrophoretic homogeneity by metal affinity chromatography [5]. The L chain (9.4 μM) was denatured by treatment in 6 M guanidine hydrochloride (GdmCl) in buffer A (50 mM Tris-HCl, pH 7.7, 0.025% Tween 20 and 0.02% sodium azide (30 min, 25°C)) and renatured by dilution with the buffer to 0.16 μM L chain and 0.1 M GdmCl, followed by dialysis (12–14 kDa cutoff) (4°C, 3 changes, 2 days; final GdmCl concentration <0.2 nM assuming complete equilibration). The control recombinant L chain incubated in buffer A without Gdmcl was subjected to identical processing. For comparison, the L chain was also prepared by reduction and alkylation of the IgG secreted by hybridoma cells (clone c23.5), followed by gel filtration in 6 M GdmCl and renaturation by dialysis [6]. Fluorescence emission spectra (λ_{em} 310 to 500 nm; λ_{ex} 293 nm) of the renatured L chain concentrated using a Centricon-10 ultrafiltration device and then diluted in buffer A or in 6 M GdmCl were obtained using a Perkin Elmer LS50 fluorimeter. Cleavage of peptide–MCA conjugates (Peptides Intl.) was measured by spectrofluorimetry in 96-well microplates [4]. The data are corrected for background fluorescence of substrate incubated in diluent without antibody.

3. Results and discussion

The L chain in 6 M GdmCl solution displayed increased peak fluorescence emission compared to the undenatured L chain (ΔFU 122) (Fig. 1). A shift in the emission maximum of the L chain in 6 M GdmCl (356 nm) was evident compared to control L chain (344 nm). After renaturation of the L chain from the GdmCl solution, a decrease in fluorescence intensity by 79 FU and a 3 nm shift in fluorescence maximum (λ_{max} 347 nm) compared to the undenatured L chain was observed, indicating the occurrence of a conformational transition due to the denaturation/renaturation treatments.

Cleavage of the following synthetic peptide substrates (50 μM) by L chains (0.4 μM) was analyzed: A–MCA, R–A–MCA, M–MCA, Cbz–V–K–M–MCA, F–MCA, L–MCA, Suc–L–L–V–Y–MCA, Suc–A–D–MCA, Boc–V–L–K–MCA, P–F–R–MCA and Boc–E–A–R–MCA. The control recombinant L chain (*rec* L chain) kept in non-denaturing solvent displayed marked selectivity for cleavage of MCA linked to R or K residues, consistent with our own observations described previously [4], and those of Matsuura and Sinohara [7] on L chains isolated from multiple myeloma patients. Suc–L–L–V–Y–MCA and L–MCA were cleaved very slowly but reproducibly (3.1 and 1.6 FU/22 h, respectively). Cleavage of the remaining substrates by *rec* L chain was undetectable (<1 FU/22h). *rec* L chain refolded from the denaturing solvent (*recld* L chain) displayed lower cleavage of substrates containing R–MCA and K–MCA bonds, and increased cleavage of L–MCA and Suc–L–L–V–Y–MCA (Table 1). Cleavage of Suc–L–L–V–Y–MCA by *recld* L chain was examined in detail. The rate of the reaction was linear as a function of increasing incubation time up to 30 h, and of increasing catalyst concentration (Fig. 2). Enhanced cleavage of this substrate by *recld* L chain relative to the undenatured *rec* L chain was evident at all catalyst concentrations and reaction time intervals studied. The initial rates of cleavage of varying Suc–L–L–V–Y–MCA concentrations (5–500 μM) by *recld* L chain (0.4 μM) were fitted to the Michaelis-Menten equation as in [4]. Apparent K_m and V_{max} values for the reaction were $(4.8 \pm 0.5) \times 10^{-6}$ M and 3.1 ± 0.4 mol/mol L chain/22 h, respectively.

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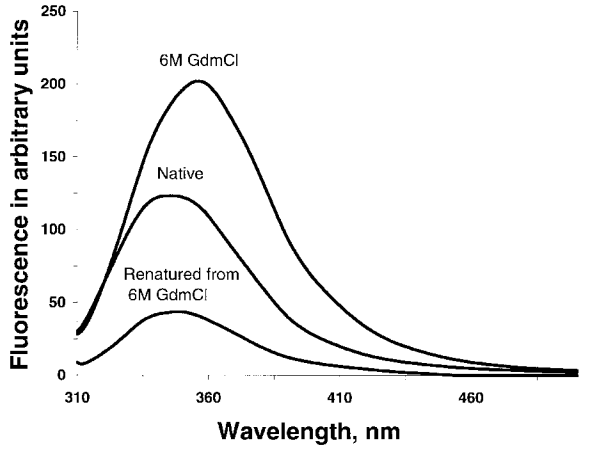

Fig. 1. Emission fluorescence spectra (310–500 nm) of recombinant L chains in aqueous buffer (native), in 6 M GdmCl, and following renaturation from the 6 M GdmCl solution (0.5 μM L chain, 0.5 ml). Excitation wavelength 293 nm.

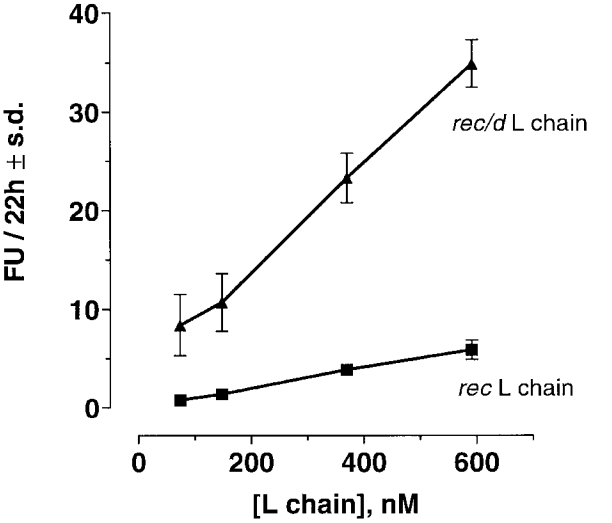

Fig. 2. Cleavage of Suc–L–L–V–Y–MCA by recombinant L chain subjected to denaturation and renaturation (*rec/d* L chain, ▲) and control recombinant L chain (*rec* L chain, ■). Substrate: 100 μM. Reaction time, 22 h.

The renatured *alk/d* L chain, which was prepared from IgG in denaturing solvent, did not display a preference for cleavage of the R–MCA bond in P–F–R–MCA (Table 1). This observation is consistent with previous results showing that *alk/d* L chain preparations cleave vasoactive intestinal peptide (the immunogen to which the L chain was raised) at Q^{16}–M^{17}, A^{18}–V^{19}, M^{17}–A^{18} and K^{20}–K^{21} bonds [6], whereas the *rec* L chain maintained under non-denaturing conditions cleaves this peptide only at K–X and R–X bonds [8]. The two forms of the L chains also bind VIP with differing affinities, evident from their K_m values [4,6]. The *alk/d* L chain has a 10-fold lower affinity for VIP [6], presumably reflecting the assumption of an improperly refolded L chain conformation.

Evidently, therefore, stabilizing interactions at positively charged R/K residues located at the cleavage site are not completely essential for catalysis by the L chain. Previous studies of proteolytic enzymes treated with solvents capable of inducing conformational perturbations [9] and of enzyme mutants with diminished ability to recognize charged amino acids in substrates [10,11] support the possibility of preparing catalysts with modified cleavage specificity. Proteolytic antibodies display remarkable specificity for individual antigens, brought about by recognition of large epitopes that include structural determinants distant from the cleavage site [5,12]. In principle, therefore, engineering of antibody active sites with redirected cleavage specificity and without loss of substrate specificity should be achievable.

Table 1
Cleavage selectivity of control recombinant L chain kept in non-denaturing solvent (*rec* L); recombinant L chain subjected to denaturation and renaturation (*rec/d* L chain); and renatured alkylated L chain prepared by fragmentation of IgG and separation from the H chain in denaturant (*alk/d* L chain)

Catalyst	Selectivity Index ± (SEM)	
	R–MCA/Y–MCA	R–MCA/L–MCA
rec L chain	14.3 ± 2.9	27.6 ± 7.1
rec/d L chain	0.4 ± 0.2	0.7 ± 0.3
alk/d L chain	1.2 ± 0.1	2.3 ± 0.2

Selectivity indices are the ratios of cleavage at the indicated bonds by L chains (0.4 μM) in FU/22 h. Substrates for measurement of cleavage at R–MCA, Y–MCA and L–MCA were, respectively: P–F–R–MCA, Suc–L–L–V–Y–MCA and L–MCA. Values of cleavage of P–F–R–MCA by the *rec* L chain, *rec/d* L chain and *alk/d* L chain were, respectively: 44.2 ± 2.6, 7.5 ± 3.2 and 5.5 ± 0.2 FU/22 h. Data are from three independent experiments.

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