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Removal of catalytic activity by EDTA from antibody light chain

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Abstract

Gp41 peptide antigen of the HIV-1 envelope (TP41-1:TPRGPDRPEGIEEEGGERDR, a highly conserved region) was enzymatically degraded by the antibody light chain 41S-2-L after an induction period. The peptide bond between Glu^{14} and Gly^{15} was cleaved early in the reaction. When EDTA was added in the induction period, it inhibited the degradation of TP41-1 thus ceasing the catalytic activity of 41S-2-L. In contrast, when EDTA was added after the induction period, only a small reduction in the catalytic activity was observed. These observations suggest that metal ions are important in stimulating catalytic activity early in the reaction.

Introduction

In the past decade and a half, many catalytic antibodies have been discovered using methods such as: 1) the immunization of transition state analogue molecules (Tramontano *et al.* 1986; Pollack *et al.* 1986; Iverson & Lerner 1989; Cochran & Schultz 1990; Thomas 1994), 2) direct immunization of ground-state peptide antigens (Paul *et al.* 1989; Paul *et al.* 1992; Sun *et al.* 1994; Uda *et al.* 1998; Hifumi *et al.* 1999; Hifumi *et al.* 1999; Uda *et al.* 2000), 3) the production of idiotypic anti-monoclonal antibodies to enzymes (Joron *et al.* 1992), and 4) the isolation of Bence Jones proteins (Paul *et al.* 1995; Matsuura *et al.* 1996; Matsuura *et al.* 1998; Sinohara & Matsuura 1999) or autoantibodies from the patients (Matuura *et al.* 1998; Li *et al.* 1995; Gabibov *et al.* 1994). These catalytic antibodies, except for the ones prepared by using transition state analogue molecules, have become well accepted as natural catalytic antibodies. Most of them are able to catalytically convert their antigens into small molecules with high activity. Interestingly, it has been demonstrated that the catalytic activity sites of anti-VIP antibody (Sun *et al.* 1994) and human immunodeficiency virus (HIV) gp41 (Hifumi *et al.* 1999) reside in the light chains of the antibodies. Bence Jones protein which is a human antibody light chain also displayed the amydolytic activity. Apparently, the enzymatic activity of catalytic antibody light chains is inherently encoded in the germ line (Kohler & Paul 1998).

It is well known that the HIV-1 envelope protein (*env*), gp41, plays an important role in HIV-1 entry to human leukocytes. Although most *en*v-encoding sequences mutate frequently, the a.a. sequence region: RGPDRPEGIEEEGGERDRD in gp41 (amino acid numbers 732–750 in gp160 of HIV-1) is highly conserved among many HIV-1 strains. This peptide is responsible for eliciting neutralizing antibodies during HIV infection (Kennedy *et al.* 1986; Vella *et al.* 1993). As we reported previously (Uda *et al.* 1998; Hifumi *et al.* 1999; Hifumi *et al.* 1999; Hifumi *et al.* 2000; Uda *et al.* 2000), our antibody light chain (41S-2-L), which was isolated from the monoclonal antibody 41S-2 following selection for its specificity to the highly conserved sequence of HIV-1 gp41 (RGPDRPEGIEEEGGERDRD), is unique in that it can enzymatically decompose not only the above target peptide but also the intact gp41 molecule with a high activity comparable to trypsin. The 41S-2-L enzymatic activity functions similarly to an endopeptidase. Though we are extensively investigating the biochemical and immunological features of the catalytic antibody, other aspects of the antibody remain unclarified. In about

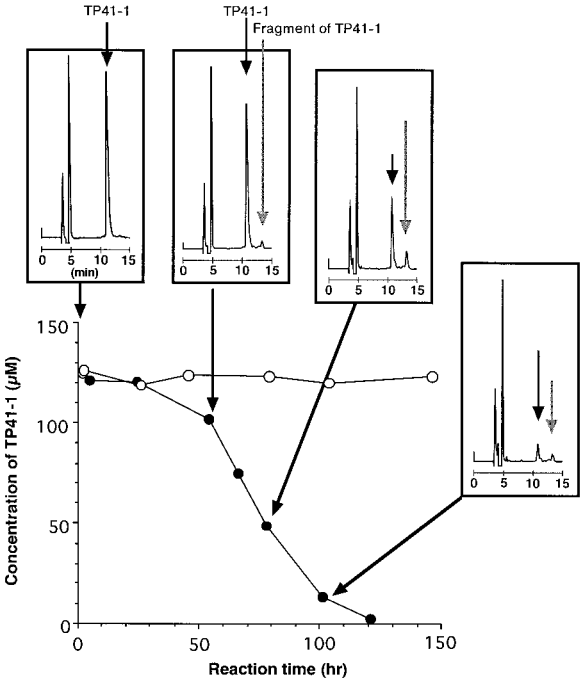

Figure 1. Catalytic reaction of gp41 peptide antigen (TP41-1) and catalytic antibody 41S-2-L. ●: TP41-1 + 41S-2-L; ○: TP41-1. TP41-1 peptide: 120 μM, catalytic antibody 41S-2-L: 0.8 μM. The reaction was carried out at 25 °C in phosphate buffer. Four boxes illustrate the results of HPLC with an increase of the reaction time.

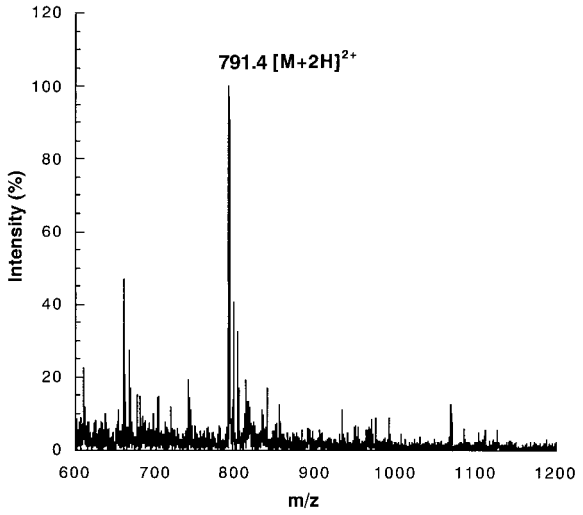

Figure 2. Mass spectrum for the fraction of the peak at 13.5 min retention time in HPLC. The fragment was identified to be TPRGPDRPEGIEE from the monoisotopic mass m/z = 791.4 ($[M+2H]^{2+}$ form). The fraction from the 13.5-min peak was collected and subjected to ion-spray type mass spectrometry in the positive ion mode with an orifice voltage of 85 volts (API-III, Perkin-Elmer Sciex, Ontario, Canada). Mass calibration was conducted using a mixture of polypropylene glycol at different molecular weights of 425, 1000 and 2000.

one third of natural enzymes, metal ions stimulate enzyme activity and/or stabilize their conformations. In the studies of catalytic antibodies, the effect of metal ions on the catalytic activity has not been clarified. Thus, in this study, the effect of metal ions was investigated by the addition of EDTA to the reaction system.

Materials and Methods

Reagents

EDTA·2Na and KCl, NaCl, $Na_2HPO_4{\cdot}12H_2O$, KH_2PO_4, 2-mercaptoethanol and guanidine hydrochloride were purchased from Wako Pure Chemicals Ltd. (Osaka, Japan). Bio-Rad Protein A MAPS II (Nippon BIO-RAD, Tokyo, Japan) was used in the affinity chromatography to purify the antibody from ascites fluid.

Purification of the antibody and isolation of the light chain

The 41S-2 monoclonal antibody (41S-2 mAb) used in this study was raised against a 19 mer peptide RGPDRPEGIEEEGGERDRD. 41S-2 mAb was purified from ascites fluid using affinity chromatography (MAPSII, BIO-Rad). The purified 41S-2 mAb was dissolved in 2.7 ml of a 50 mM Tris and 0.15 M NaCl buffer (pH 8) and then reduced with 2-mercaptoethanol for 3 h at 15 °C. To the resulting solution, 3 ml of 0.6 M iodoacetamide were added to prevent re-association of the separated subunits by autooxidation, and the pH was re-adjusted to 8 with 1 M Tris. Then, the solution was incubated for 15 min at 15 °C. After the resultant solution was concentrated to 0.5 ml by ultrafiltration, it was injected into a size exclusion chromatography column (Protein-Pak 300, 7.8 × 300 mm, Nippon Waters, Tokyo, Japan) at a flow rate of 0.15–0.20 ml/min with 6 M guanidine hydrochloride as the eluent. The fraction corresponding to the light chain was collected, diluted with 6 M guanidine hydrochloride, and then dialyzed against PBS at 4 °C. The buffer was replaced seven times for 3–4 days. The solution was also dialyzed against phosphate buffer for four days at 4 °C. The concentration of 41S-2-L was determined by a DC protein assay (Bio-Rad).

Peptide synthesis

The peptide, TP41-1 (TPRGPDRPEGIEEEGGERDRD), used in this experiment was synthesized by the Fmoc solid-phase method with an automated peptide synthesizer (Applied Biosystems 431A, CA, USA). After deprotection of the synthesized peptide from the resin, the peptide was purified using reverse-phase HPLC (RP-HPLC; Waters 490E, Waters μBONDASPHERE C_{18} column; Waters, NY, USA). The purity of the peptide was confirmed at $>$ 99% by HPLC. Peptide was identified using an ion-spray type mass spectrometer (API-III, Perkin-Elmer Sciex, Ontario, Canada).

Catalytic reaction

Prior to carrying out the catalytic reaction, most glassware, plastic ware and buffer solutions used in this experiment were sterilized by heating (180 °C, 2 h), autoclaving (121 °C, 20 min). Buffers were passed through a 0.20 μm sterilized filter. Experimental manipulations were performed in a safety cabinet to avoid air contamination.

The catalytic reaction involving 41S-2-L was carried out in 15 mM phosphate buffer (pH 6.5) at 25 °C. To monitor the reaction, 20 μl of the reacting solution was injected into a RP-HPLC column (Waters 600S, USA or Jasco, Tokyo, Japan) at room temperature under isocratic conditions.

Results

Degradation of gp41 peptide antigen (TP41-1)

The degradation of gp41 peptide antigen (TP41-1:TPRGPDRPEGIEEEGGERDRD) by the antibody light chain 41S-2-L was carried out in phosphate buffer (pH 6.5). The reaction profile is shown in Figure 1. In agreement with previous findings, degradation of the TP41-1 peptide by freshly-prepared 41S-2-L displayed a double phase reaction profile (Uda *et al.* 1998; Hifumi *et al.* 1999). Up to about 50 h, the degradation progressed slowly (i.e. the first phase: induction period). The reaction rate increased abruptly after 50 h (i.e. the second phase: highly active period). As illustrated by the results of HPLC, TP41-1 peptide (the retention time is 11 min) decreased with increasing reaction time (Figure 1). In contrast, the peak at a retention time of 13.5 min increased at first, and then decreased as the reaction time elapsed. At 100 h, both peaks became small and finally disappeared by 120 h. The fraction corresponding to the peak at 13.5 min was collected and subjected to mass spectrometric analysis (Figure 2). The obtained spectrum indicated that the fraction had a monoisotopic mass of 1580.8 (calculated using the m/z = 791.4 ($[M+2H]^{2+}$ form)). The obtained mass was consistent with that of the TPRGPDRPEGIEEE peptide (1580.8). These results suggest that 41S-2-L hydrolyzed the peptide bond between Glu^{14} and Gly^{15} in the a.a. sequence: TPRGPDRPEGIEEEGGERDRD. The other peptide fragment, GGERDRD, could not be identified likely because of its low concentration or early retention time. Amino acid analysis revealed many amino acids in the final product solutions after complete degradation of TP41-1. As some unidentified peaks were also observed in the chromatogram of the analysis (data not shown), it is plausible that di- or tri-peptides co-existed in the final products.

Effect of EDTA in the catalytic reaction with 41S-2-L

EDTA was added to the reaction mixture at concentrations ranging from 2 to 200 μM. EDTA suppressed the catalytic activity of 41S-2-L in a concentration dependent manner (Figure 3). Higher EDTA concentrations had stronger inhibitory effects. At greater than 30 μM EDTA, catalytic activity ceased. As expected, in solutions of 30 μM of EDTA and greater, the difference in inhibitory effects between respective EDTA concentrations became very small as described below.

Inhibitory effects due to different times of EDTA addition

In this set of experiments, the degradation of TP41-1 without EDTA occurred in a manner similar to that shown in Figure 1 and 3, but with a faster completion time. This was probably caused by a difference in the amount of 41S-2-L in the prepared lots. The catalytic activity of 41S-2-L fluctuates slightly among the lots. Thirty μM of EDTA might be the lowest concentration for complete inhibition of the catalytic activity of 41S-2-L. Hence, it is speculated that very small quantities of 41S-2-L remained in the native form, thus displaying small activity levels upon the addition of EDTA at 0 and 12 h. Regardless, the addition of EDTA at 0 and 12 h (induction period), resulted in strong suppression of TP41-1 degradation. However, addition of EDTA at 24, 36, 48, 53 and 57 h (highly active period), yielded lower efficiency of catalytic activity inhibition than addition during the inhibition period. Therefore,

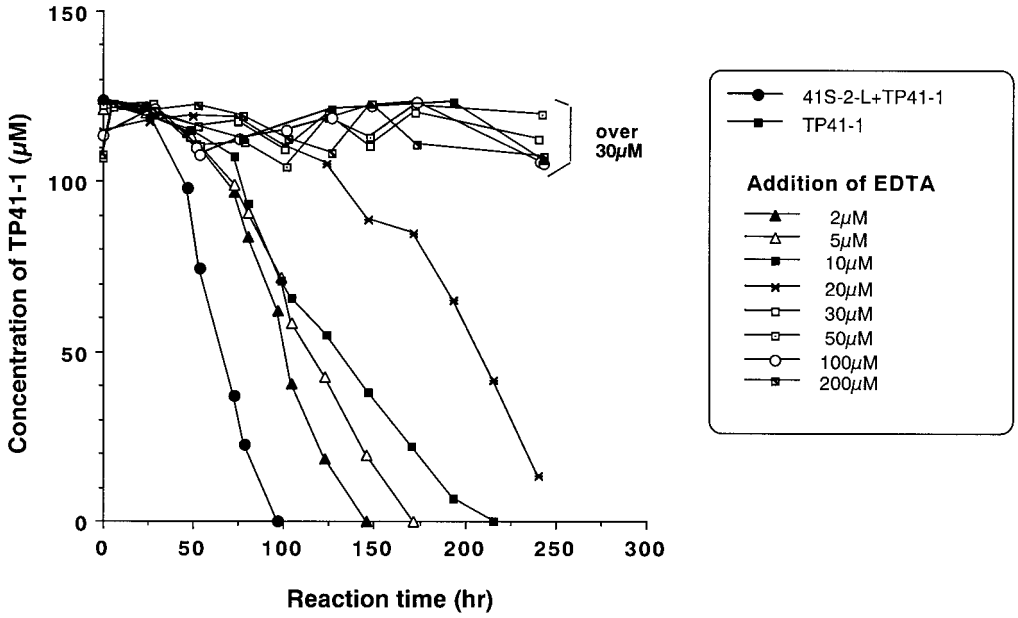

Figure 3. Effects of EDTA on the catalytic activity of 41S-2-L. The reactions were performed under the same conditions as in Figure 1. 41S-2-L: 0.8 μM, TP41-1: 120 μM.

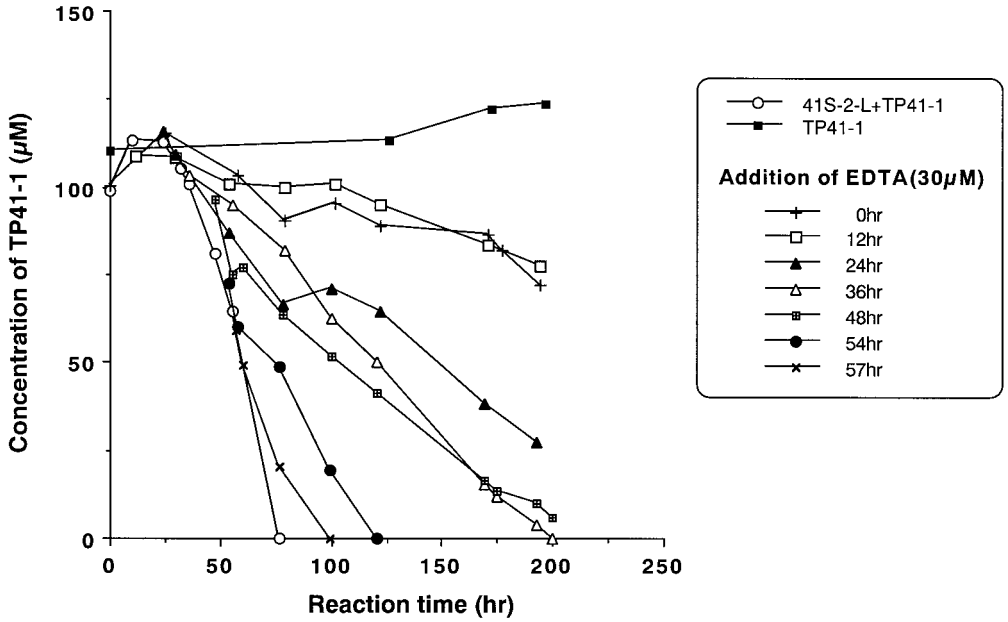

Figure 4. Inhibitory effects from different times of EDTA addition. The reactions were performed under the similar conditions as in Figure 1. 41S-2-L: 0.72 μM, TP41-1: 108 μM, EDTA: 30 μM.

suppression of the catalytic activity was dependent on EDTA addition time.

Discussion

Taken together, results of Figure 1 and 2 and the references cited above clearly indicate that TP41-1 is consecutively converted into small molecules via the intermediate, TPRGPDRPEGIEEE. The cleaved peptide bond is between Glu^{14} and Gly^{15} in phosphate buffer (pH 6.5).

Recall that the decrease in TP41-1 levels did not correspond to the generation of fragments bearing a retention time of 13.5 min (Figure 1). The antibody light chain 41S-2-L has a high specificity to gp41 molecule (41 kDa) as it is a monoclonal antibody subunit. However, the smaller the antigen molecule is, the lower the specificity of 41S-2-L becomes. Therefore, we postulate that 41S-2-L functions as a catalyst via 2 sites. One site specifically binds to the antigen (antibody feature), while the other functions to catalytically cleave the peptide bond of small peptides as well as antigenic molecules (enzymatic feature). If we consider that the peptide bond is cleaved by the catalytic triad that is mainly expressed from the CDR-2 and FR-3 sequence of 41S-2-L (Hifumi *et al.* 2000), consecutive degradation of the intermediate product, TPRGPDRPEGIEEE, could advance by a catalytic triad which is not highly specific to the antigen.

It is very interesting that the addition of EDTA exhibited a huge effect on the catalytic activity of 41S-2-L, whereby its inhibitory effect was concentration-dependent. In solutions of greater than 30 μM EDTA, 41S-2-L completely failed to degrade TP41-1. Which molecule was affected by EDTA; TP41-1 or 41S-2-L ? In the starting solution, 120 μM of TP41-1 and 30 μM of EDTA co-existed. Even if all EDTA could quantitatively eliminate all the metal ion bound to TP41-1, a considerable amount of TP41-1 would still remain in its native form. Therefore, it is highly unlikely that the inhibition effects observed in the 30 μM EDTA solutions were due to an interaction of EDTA with TP41-1 peptide. As 41S-2-L was present at a concentration of only 0.8 μM in the starting solution, it is reasonable that EDTA acts on 41S-2-L instead of the TP41-1 peptide.

In order to quantitatively interpret the results of Figure 4, the time it took one half of the TP41-1 to be hydrolyzed was estimated. This time represents the relative inhibitory effect of EDTA on the catalytic activity of 41S-2-L. The results are presented in Figure 5. For EDTA addition at 0 and 12 h (induction period), it took 300 and 310 h for the hydrolysis of one half of TP41-1, respectively. However, for addition at 24, 36, 48, 53 and 57 h, it took much less time (i.e. 106, 84, 72, 36 and 15.5 h; respectively). Therefore, EDTA suppresses effectively the catalytic activity of 41S-2-L, when it is added in the induction period. In contrast, inhibition effects of the catalytic activity are low when EDTA is added after the induction period. Conformational change of 41S-2-L was not examined by CD spectroscopy, because the large amount of TP41-1 peptide (120 μM) masked the spectrum of the much smaller amount of 41S-2-L (0.8 μM). However, by using fluorescent spectroscopy, we have already pointed out that the conformational change of 41S-2-L must take place during the induction period (Uda *et al.* 2000). Taken together, these data suggest that the metal ion does not participate in the structure of the active site, but accelerates the crucial conformational transition of 41S-2-L in the induction period. Apparently, 41S-2-L can not acquire catalytic activity if the metal ion is eliminated from 41S-2-L by EDTA in the early stage of the reaction. The effective metal ions were ferric, zinc and calcium. Addition of ferric

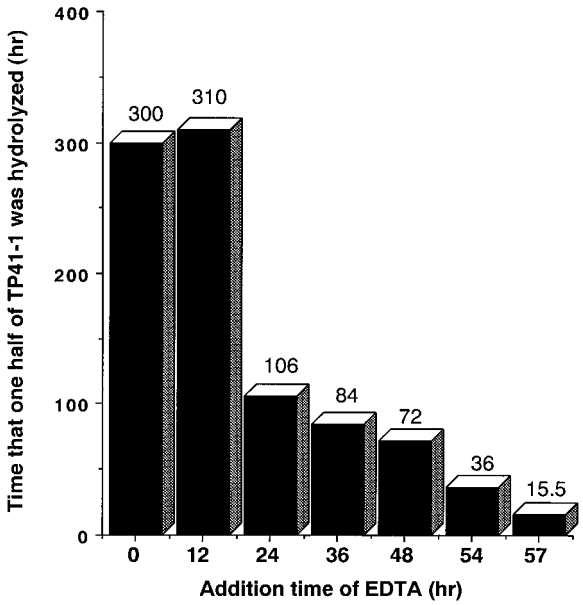

Figure 5. Relation between the addition time of EDTA and the time it took one half of the TP41-1 to be hydrolyzed. For the addition at 0 and 12 h, the time that one half of TP41-1 was hydrolyzed was estimated to be 300 and 310 h by extrapolating the degradation lines in Figure 4. Others were derived by interpolating the curves in Figure 4.

ion yields complete recovery of the catalytic activity of 41S-2-L as will be reported in the near future.

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