# Isomerization of an Aspartic Acid Residue in the Complementarity-Determining Regions of a Recombinant Antibody to Human IgE: Identification and Effect on Binding Affinity

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ABSTRACT: This report describes the effect on antigen binding of an isomerized aspartate residue located in the complementarity-determining regions (CDRs) of a recombinant monoclonal antibody. The antibody, which binds human IgE, contains two Asp-Gly sequences within its CDRs, but only one site was found to be labile to isomerization. Isolation and characterization of antibody fragments differing in the labile sequence were facilitated by using a technique involving hydrophobic interaction chromatography (HIC) that separates aspartyl, isoaspartyl, and cyclic imide variants to the residue located in CDR-L1. The variants were isolated for structural characterization and for determination of their relative antigen binding affinities. Mutants were constructed with altered residues to obviate the effects of isomerization and were evaluated for their ability to bind to IgE. Inspection of published crystal structures of CDRs of antibodies indicated that hydrogen binding of the Asp side chain of the unreactive residue may be the constraint that prevents isomerization. The strategy outlined here may prove to be of general utility in the biochemical and immunochemical characterization of recombinant antibodies.

Monoclonal antibodies (MAbs) are characterized by highly selective binding of a target antigen. The selectivity can be exploited for detection of the antigen in diagnostic applications, for purification in immunochromatographic techniques, to inhibit biochemical interactions *in vitro*, or as human therapeutics. The therapeutic usefulness of MAbs can be improved in many applications by employing recombinant DNA technology to produce humanized MAbs (rhMAbs). These molecules are produced by splicing the residues from, e.g., a murine MAb that confer antigen specificity into a human immunoglobulin framework (Jones *et al*., 1986). This process yields a protein that is identical to human antibodies, save for the highly variable residues within the complementarity-determining regions (CDRs). The resulting molecule is less prone to eliciting an immunogenic response when administered to humans than are MAbs derived from other species, such as mice.

rhMAbs are subject to degradation reactions that affect all proteins, such as isomerization of aspartate residues. As shown in Figure 1, aspartate residues (I) in Asp-Gly sequences can isomerize to isoaspartate (II) through a cyclic imide intermediate (III) at an appreciable rate (Geiger & Clarke, 1987). The equilibrium, rate, and pH dependence of this reaction have been studied in model peptides separated by reversed-phase HPLC[1] (Oliyai & Borchardt, 1993). Since the reaction is reversible, and the intermediate is relatively

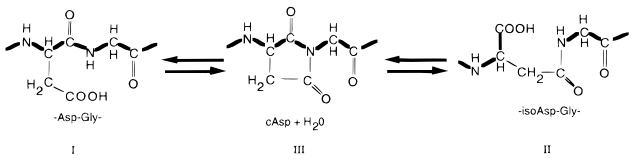


FIGURE 1: Chemical mechanism for isomerization of Asp-Gly (I) sequences. The reaction proceeds reversibly through a stable cyclic imide intermediate (III), resulting in an isoaspartic acid-containing sequence (II).

stable, peptides with labile Asp residues can be found in three forms containing either Asp, iso-Asp, or cyclic imide (cAsp) at the pertinent position. The tendency to undergo isomerization has been postulated to depend on the local flexibility of the portion of the molecule containing the Asp-Gly sequence (Geiger & Clarke, 1987). In this mechanism, sufficient local flexibility is required to accommodate the structural differences of the three species. The three forms interconvert and so approach an equilibrium that is temperature- and pH-dependent (J. Frenz and J. Cacia, in preparation).

As human therapeutics, monoclonal antibodies are subject to the same stringent oversight of their structural characteristics and consistency of manufacture as other protein pharmaceuticals. Sensitive analytical techniques are required to characterize a MAb intended for clinical uses to fully characterize its relevant features. Variants generated by spontaneous mutagenesis or degradative processes may alter the specificity and strength of antigen binding, particularly if the residue(s) involved are in the CDRs of the MAb. Variants that are silent with respect to antigen binding also present manufacturing and product consistency issues but may not directly impair the efficacy of the MAb as a drug. One example is the variable degree of post-translational processing of the C-terminal lysine in the Fc region of IgGs, which has been attributed to carboxypeptidase activity (McDonough *et al*., 1989). A second example is the recent

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[1] Abbreviations: huFcεRI, human high-affinity IgE receptor; HIC, hydrophobic interaction chromatography; iso-Asp, isoaspartate; cAsp, cyclic imide (succinimide) intermediate; HPLC, high-performance liquid chromatography; EDTA, ethylenediaminetetraacetic acid; PTH, phenylthiohydantoin.

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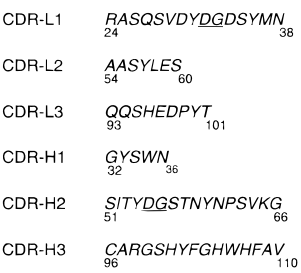


FIGURE 2: Primary structures of the complementarity-determining regions (CDRs) of the E25 rhMAb. The CDRs are defined as described in Presta *et al*. (1993).

identification of a genetic mutant of a recombinant monoclonal antibody, involving a point mutation in the Fc region of the molecule (Harris *et al*., 1993).

E25 is a rhMAb that prevents binding of IgE antibodies to high-affinity receptors (Presta *et al*., 1993) and thereby shows promise as a treatment for allergic diseases (Chang *et al*., 1990). E25 (Figure 2) contains Asp-Gly sequences in CDR-L1 at L32−33 (i.e., residues 32−33 in the light chain) and in CDR-H2 at H55−56. This report describes the evidence for the occurrence of iso-Asp and cAsp residues in E25 samples incubated at 37 °C and pH 5.2.

## EXPERIMENTAL PROCEDURES

*Materials*. Recombinant human monoclonal antibody E25 was cloned, expressed, and purified at Genentech, Inc. The antibody was prepared at 5 mg/mL in a buffer containing 10 mM acetate (pH 5) and incubated in a 37 °C water bath to generate the isomeric forms of the antibody characterized in this study. E25 mutant Fabs were prepared using site-directed mutagenesis and purified as described (Presta *et al*., 1993).

*Hydrophobic Interaction Chromatography*. Analytical HIC was performed on a 4.6 × 30 mm TSK-Butyl NPR column (Tosoh Haas, Montgomeryville, PA). Buffer A consisted of 2 M ammonium sulfate in buffer B [20 mM Tris and 20% glycerol (pH 8.0)]. The column temperature was maintained at 40 °C. Intact E25 (5 $\mu$g) was injected and eluted with a 15 min linear gradient from 60 to 35% buffer A at a flow rate of 1 mL/min. Antibody fragments of E25 were separated with a 20 min linear gradient from 90 to 38% buffer A. The HIC column was washed with 100% buffer B for 10 min following each run and equilibrated at the initial gradient conditions for 10 min prior to injection. All chromatography was performed on a Hewlett-Packard (Palo Alto, CA) 1090M HPLC system with detection by UV absorbance at 214 nm.

Preparative collection was performed on E25 Fab and F(ab′)$_2$ fragments for characterization using a TSK Phenyl 5PW column (7.5 × 75 mm). The column was run at 40 °C and 0.8 mL/min. Buffer A contained 2 M ammonium sulfate in buffer B [25 mM sodium phosphate and 20% glycerol (pH 7.0)]. A 70 min linear gradient was run from 70 to 20% buffer A. Fractions were collected manually and concentrated using Centricon-10 microconcentrators (Amicon, Beverly, MA).

*Pepsin Digestion of E25*. Samples of E25 were diluted 5-fold with 0.1 M sodium citrate (pH 3.5). Pepsin (Worthington Biochemicals, Freehold, NJ) was freshly resuspended in citrate buffer and added to E25 samples in a final pepsin:E25 ratio of 1:100 (w:w). The samples were then incubated at 37 °C for 2 h and injected into the HPLC.

*Papain Digestion of E25*. Samples of intact or F(ab′)$_2$ E25 were exchanged into papain digest buffer containing 0.1 M sodium phosphate and 2 mM ethylenediaminetetraacetic acid (EDTA) (pH 7.0) using Bio-Spin 30 columns (Bio-Rad, Hercules, CA). Freshly prepared L-cysteine was added to the samples to achieve a concentration of 10 mM. Papain (Worthington Biochemicals) was added at a papain:E25 ratio of 1:100 (w:w) to the samples which were then incubated at 37 °C for 4 h. Samples were frozen at −20 °C until Fab fragments were preparatively collected or analyzed by HIC.

*Tryptic Peptide Mapping*. S-Carboxymethylation of E25 Fab fragments was performed prior to digestion with trypsin. Samples were concentrated and exchanged using Centricon-30 microconcentrators (Amicon) into a solution containing 8 M urea, 0.35 M Tris, and 1 mM EDTA at pH 8.3. Digestion and mapping conditions were essentially as described (Harris *et al*., 1993). Peak fractions were collected manually.

*Liquid Chromatography−Mass Spectrometry (LC−MS)*. Mass spectrometric analyses were performed using a Sciex (Thornhill, Ontario) API III instrument operating in the positive-ion mode. The HPLC solvents A and B were 0.1% trifluoroacetic acid (TFA) in water and 0.1% TFA in acetonitrile, respectively. The E25 Fab fragments were injected onto a TSK-Phenyl 5PW column equilibrated with 10% solvent A at 45 °C with a flow rate of 0.5 mL/min. A linear gradient was run to 60% solvent A in 40 min. The column eluate was split at a ratio of 1:100 and introduced into the mass spectrometer at a flow rate of 10 $\mu$L/min.

*N-Terminal Sequence Analysis*. The cyclic imide intermediate was identified and sequenced using a previously described procedure (Kwong & Harris, 1994). E25 Fab tryptic fragments were sequenced on an Applied Biosystems (Foster City, CA) Model 477A/120A system. Molar values for the PTH amino acids were generated by peak area comparisons to an external standard mixture.

*IgE Receptor Binding Inhibition Assay*. The biological activity of E25 variant fragments and site-directed mutants was assessed by measuring their respective abilities to inhibit the binding of human IgE to the human high-affinity IgE receptor, huFc$\epsilon$RI, as described previously (Presta *et al*., 1993). An intact E25 standard was used to quantify samples.

## RESULTS

### *HIC Separation of E25 Isoforms*

The hydrophobic interaction chromatography conditions—including column choice, buffer composition, and pH and column temperature—were optimized for resolution of isoforms of intact E25, as described elsewhere (J. Frenz, J. Cacia, and R. Keck, in preparation). The chromatogram of E25 incubated at 37 °C for 21 days, shown in Figure 3, contains seven peaks that represent forms of the antibody containing combinations of heterogeneities at different sites on the bivalent antibody, as detailed below. One source of heterogeneity is the partial processing of a C-terminal lysine on the heavy chain. This can be shown by treatment of the antibody with carboxypeptidase B, which cleaves basic residues from the C-terminus of proteins and eliminates peaks

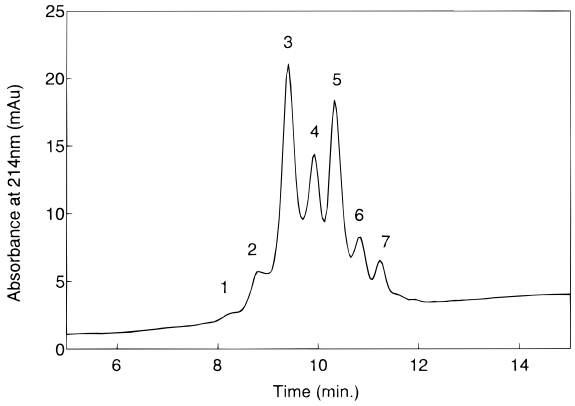


FIGURE 3: Separation by hydrophobic interaction chromatography of intact E25. Peaks labeled 1−7 correspond to the variants identified in Table 1. Separation conditions are described in the text.

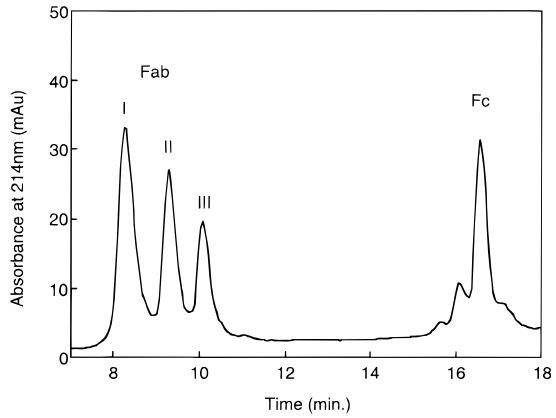


FIGURE 4: Separation by hydrophobic interaction chromatography of the mixture resulting from papain digestion of E25. The portion of the chromatogram labeled Fab includes the three heterodimeric fragments containing either Asp, iso-Asp, or cAsp at position 32. The portion labeled Fc includes the homodimeric Fc fragments that contain one or both of the unprocessed C-terminal lysine residues, as well as the major constituent, the fully processed fragment. The separation conditions are described in the text.

1 and 2 from the chromatogram, without affecting the positions of peaks 3−7 (data not shown). The remaining source of heterogeneity in these peaks was identified as described below.

*Characterization of E25 Isoforms*

*Papain Digestion and HIC Separation of C-Terminal Heterogeneity*. A convenient way to localize the sources of heterogeneities on an antibody molecule is by selective protease digestion followed by separation of the resulting fragments. Both papain and pepsin digestion followed by HIC separation were employed to characterize the heterogeneity observed in the E25 antibody. Papain cleaves the E25 molecule on the C-terminal side of His-H228 to yield two Fab fragments and an Fc fragment. The digest mixture can be resolved on an HIC column as shown in Figure 4. Two peaks in the chromatogram yielded masses that correspond to the expected mass of the Fab fragment, 48 194, by LC−MS, as shown in Table 1. The third peak yielded a mass 16 amu smaller. The Fc fragment was resolved into three peaks, but treatment of the antibody with carboxypeptidase B prior to papain digestion (data not shown) results in disappearance of the two peaks eluting slightly ahead of

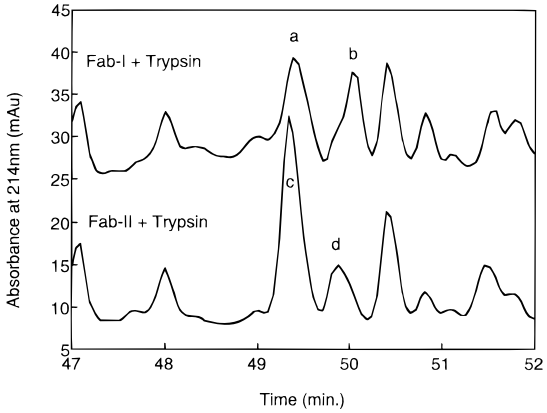


FIGURE 5: Portions of the chromatograms of mixtures resulting from trypsin digestion of purified Fab-I and Fab-III E25 variants. This portion of the chromatogram contains the four peaks labeled a−d that were collected and subjected to N-terminal sequence analysis. The reversed-phase HPLC separation of the digest mixture was carried out as described in the text.

Table 1: Mass Spectrometric Analyses of E25 Fab Variants

| Fab sample | expected mass | observed mass | identity |
|---|---|---|---|
| Fab-I | 48 194 | 48 194 | L1−217, H1−228 (Asp @ LC-32) |
| Fab-II | 48 194 | 48 194 | L1−217, H1−228 (iso-Asp @ LC-32) |
| Fab-III | 48 176 | 48 178 | L1−217, H1−228 (cAsp @ LC-32) |

Table 2: N-Terminal Sequence Results of E25 Fab Tryptic Peptides

| peak | sequences observed | identity |
|---|---|---|
| a | 40 pmol of GPSVFPLAPSSK | H128−139 |
| | 2.5 pmol of DYFPEP | H154−159 |
| b | 5.0 pmol of ASQSVDYDGDSYMNW | L25−46 (Asp @ 32) |
| | 5.0 pmol of NTFYLQMNSLR | H77−87 |
| | 1.5 pmol of LLIY | L50−53 |
| c | 6.0 pmol of ASQSVDY[a] | L25−46 (iso-Asp @ 32) |
| | 75 pmol of GPSVFPLAPSSK | H128−139 |
| | 3.0 pmol of DYFPEP | H154−159 |
| d | 10 pmol of NTFYLQMNSLR | L50−53 |
| | 2.0 pmol of LLIY | H77−87 |

[a] Sequence reaction terminated.

the main peak. Hence, these peaks represent forms of the antibody that retain either or both of the C-terminal lysines. This procedure thus provides a means for quantification of the extent of processing of these residues; in this case, about 3.5% of the E25 molecules contains both lysine residues, 16.5% contains a single lysine, and 80% lacks both residues.

*Characterization of Fab Fragments*. The identities of the Fab fragments were established as follows. The peaks identified in Figure 4 as Fab-I and Fab-II, which have the mass expected of the correct Fab fragment (Table 1), were individually collected and digested with trypsin and the resulting peptides separated by reversed-phase HPLC. The tryptic maps for the two forms of Fab were essentially identical except for the portions compared in Figure 5. The four peaks labeled a−d were collected and subjected to mass spectrometry and 15 cycles of N-terminal sequencing. Mass spectrometric analysis of the collected fractions indicated that peaks b and c both contained a peptide with the mass expected (2568 amu) for the full length L25−46 peptide that contains the labile Asp-L32 residue. The sequences obtained for the four peaks are shown in Table 2 and reveal that peak b from the map of the Fab-I fragment contains the full length unmodified L25−46 peptide (Table 2). Peak c, in the map

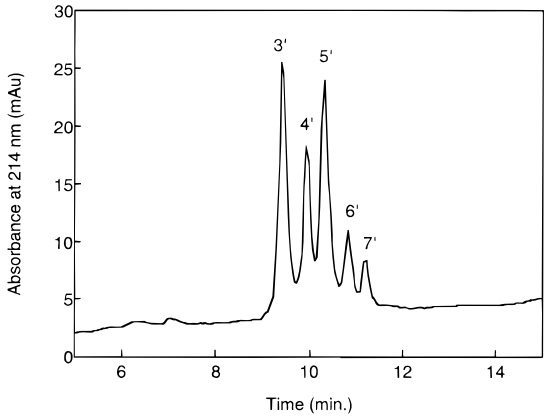


FIGURE 6: Separation by hydrophobic interaction chromatography of the mixture resulting from pepsin digestion of E25. The peaks labeled 3′−7′ correspond to the variant identities summarized in Table 3. The separation conditions are described in the text.

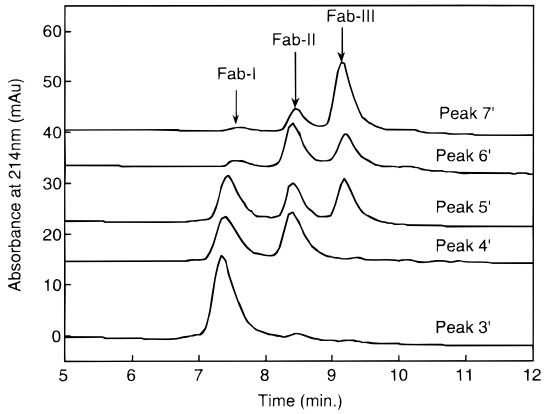


FIGURE 7: Separation of mixtures generated by papain digestion of F(ab′)$_2$ variants of E25. Peaks 3′−7′, shown in Figure 6, were individually collected, subjected to papain digestion and separated under the same conditions as in Figure 4, resulting in the three Fab fragments labeled Fab-I, Fab-II, and Fab-III.

of the Fab-II fragment, contains a peptide beginning with Ala-L25 along with the two peptides found in peak a. Termination of the sequencing reaction at cycle 6, just before an expected Asp residue in a peptide that mass spectrometry indicates is the full length L25−46 tryptic peptide, suggests that the Asp-L32 residue has isomerized to isoaspartate (Allen, 1981). Isomerization of the Asp residue does not change the mass of the peptide. Hence, Fab-I is the fragment containing the expected Asp-L32 residue, and Fab-II is the fragment with the isoAsp variant.

The Fab-III fragment was found by LC−MS to have a mass of 49 178 (Table 1). This mass is in close agreement with the value of 48 176 expected of the succinimide intermediate of the isomerization reaction, according to the scheme shown in Figure 1. The identity of this fragment as the cyclized aspartate (cAsp) form of the Fab was consistent with its observed rapid conversion to a mixture of Fab-I and Fab-II under mild conditions (data not shown). This assignment was further supported by a procedure that has been described for direct identification of cyclic imide intermediates in proteins (Harris & Kwong, 1994). HIC peaks Fab-I and Fab-III were collected and subjected to this procedure in which hydroxylamine is used to cleave the protein at succinimide residues. Fab-I yielded only the expected N-terminal sequences of the heavy and light chains after hydroxylamine treatment (data not shown). Fab-III, on the other hand, gave a sequence beginning with Gly-L33 in addition to the heavy and light chain sequences, consistent with the chain cleavage of the cyclic imide at Asp-L32 that occurs upon treatment with hydroxylamine.

Hence, these results demonstrate the occurrence of the isomerization products in CDR-L1 of the E25 antibody and the assignment of Fab-I as the Asp-containing fragment, Fab-II as the iso-Asp-containing fragment, and Fab-III as the cAsp form.

*Pepsin Digestion Followed by HIC Separation*. Two sources of heterogeneity were identified by papain mapping: partial processing of the C-terminal lysine on each of the heavy chains and isomerization of Asp-L32. To demonstrate that these two features account for the occurrence of seven peaks in the E25 chromatogram shown in Figure 3, digestion with pepsin was employed. This treatment cleaves the Fc portion of the antibody, removing C-terminal heterogeneity. The resulting mixture can be separated into five components by HIC, as shown in Figure 6, that correspond with peaks 3−7 in the chromatogram of intact E25 shown in Figure 3. The F(ab′)$_2$ fragments are better resolved on the HIC column, and so these were used rather than the intact antibody for characterization of the combinations of variants found in E25. The F(ab′)$_2$ peaks are labeled 3′−7′ to emphasize their relationship to the separation of the components of intact E25.

*Papain Digestion and CDR Mapping of F(ab′)$_2$ Fragments*. Each of the F(ab′)$_2$ peaks in Figure 6, denoted 3′−7′, was individually collected and subjected to digestion with papain in order to identify differences among the species. Papain digestion of each of the bivalent F(ab′)$_2$ variants yields two antigen binding fragments that can be resolved by HIC. Figure 7 shows chromatograms identifying each of the Fab fragments released by digestion of each of the peaks collected from the chromatogram shown in Figure 6. The papain digest of peak 3′ comprises only the Fab-I variant; that is, it contains the expected Asp-L32 residue in both antigen binding sites. Peak 4′ yields roughly equal amounts of Fab-I and Fab-II and so has Asp-L32 in one binding site and iso-Asp-L32 in the other binding site of the antibody, while peak 5′ shows all three forms of Fab in the papain map. Peak 6′ yields mainly Fab-II and Fab-III in an approximately 2:1 ratio, with only trace amounts of Fab-I. Peak 7′ yields mainly Fab-III, with much smaller amounts of the other two species. The assignments of identities to peaks 5′−7′ are complicated by the relatively rapid hydrolysis of cAsp residues under the conditions employed for the papain digestion. Fab-III is the isoform containing cAsp-L32 and appears in the papain maps of peaks 5′−7′. Hydrolysis of this residue to Asp and iso-Asp must be taken into account in order to interpret the papain maps of these fragments. Hence, peak 5′ represents the species of antibody with Asp-L32 in one antigen binding site and cAsp-L32 in the other. By this reasoning, the appearance of a relatively small amount of the iso-Asp form of the Fab following digestion is due to hydrolysis of the cAsp form, Fab-III. Peak 6′ represents the coelution of antibody fragments containing iso-Asp in both binding sites and the isoform with iso-Asp in one site and cAsp in the other. Finally, peak 7′ consists of the fragment with cAsp in both antigen binding domains. Hence, this analysis distinguishes those isoforms that are unmodified (peak 3′) from those with one unmodified antigen binding site (peaks 4′ and 5′) and from those that are

Table 3: Identities and Binding Affinities of E25 Isoforms Resolved by HIC

| peak ID[a] | F(ab′)$_2$ peak ID[b] | number of Lys residues at position H451[c] | residues at L-32 sites in bivalent antibody[d] | relative F(ab′)$_2$ binding affinity[e] |
|---|---|---|---|---|
| 1 | NA | 2 | Asp-Asp | NA |
| 2 | NA | 1 | Asp-Asp | NA |
| 3 | 3′ | 1 | Asp-Asp | 100 ± 3.8 |
| 4 | 4′ | 0 | Asp-iso-Asp | 42 ± 1.5 |
| 5 | 5′ | 0 | Asp-cAsp | 51 ± 0.6 |
| 6 | 6′ | 0 | iso-Asp-iso-Asp<br>iso-Asp-cAsp | 15 ± 0.5 |
| 7 | 7′ | 0 | cAsp-cAsp | 22 ± 0.7 |

[a] HIC peak number of the intact antibody, as shown in Figure 3. [b] HIC peak number of the F(ab′)$_2$ antibody fragment, as shown in Figure 5. NA = not applicable owing to removal of Fc fragment by pepsin. [c] 2 indicates the presence of both Lys-H451 residues on the antibody, 1 indicates a single remaining Lys residue, and 0 denotes that both Lys residues have been removed from the molecule. [d] Entries refer to the principal variant occurring in residue 32 in each of the light chains. The two entries indicate the two individual light chains in the bivalent antibody. Asp = aspartate, iso-Asp = isoaspartate, and cAsp = cyclic imide intermediate in the conversion between Asp and iso-Asp. [e] Activity measurements are normalized to the native Asp-Asp form of the antibody.

modified in both sites (peaks 6′ and 7′). The assignments of the seven peaks in the chromatogram of E25 shown in Figure 3 can be made by analogy to the results obtained on pepsin-digested antibyd and are summarized in Table 3 along with the identities f the F(ab′)$_2$ variants. The assignments in Table 3 only show the principal components of each of the peaks of the intact antibody, since there are expected to be coelutions of minor components that sport different combinations of light chain Asp-L32 and heavy chain Lys-H451 heterogeneity.

### *In Vitro Activity of E25 F(ab′)$_2$ Variants*

The effect of isomerization of Asp-L32 on the affinity of E25 for IgE was assessed by measuring the ability of F(ab′)$_2$ variants purified by HIC to inhibit binding of IgE to its high-affinity receptor. Peaks 3′−7′ from a separation such as shown in Figure 6 were collected and tested for their relative ability to block binding of IgE to the receptor, huFc$\epsilon$RI, with results shown in Table 3. The singly modified forms of the antibody (peaks 4′ and 5′) have roughly half the binding affinity for IgE as the native form of the antibody (peak 3′). Furthermore, when the antibody is modified at both functional valences (peaks 6′ and 7′), the relative binding affinity is again halved with respect to the singly modified species.

### *Interaction of E25 Variants with IgE*

The effect of variations in the Asp-L32 residue on the affinity of E25 for IgE was further assessed by quantification of the ability of Fab variants to inhibit binding of IgE to its receptor. The residues important in affecting the affinity of E25 for IgE were previously defined (Presta *et al*., 1993). This study included Fab fragments that were prepared by site-directed mutagenesis of the labile Asp-L32 and nearby residues. The mutants were used to probe the importance of various residues, including Asp-L32, in IgE binding and to examine the feasibility of stabilizing the molecule with respect to isomerization while maintaining antigen recognition. The results, summarized in Table 4, indicate that changing Asp-L32 decreased the relative binding affinity for

Table 4: Identities and Relative Binding Affinities for rhuMAb-E25 Fab Variants and Mutants

| Fab variant or mutant | identity (variation from sequence shown in Figure 2) | relative binding affinity[a] |
|---|---|---|
| E25 Fab-I | CDR sequences shown in Figure 2 | 100 ± 3.9 |
| E25 Fab-II | Asp-L32 → iso-Asp | 13 ± 0.7 |
| E25 Fab-III | Asp-L32 → cAsp | 16 ± 2.6 |
| V10 | Asp-L30 → Ala<br>Asp-L32 → Ala<br>Asp-L34 → Ala | <1.0 |
| V100 | Asp-L32 → Ala | 27 ± 2.7 |
| V103 | Asp-L32 → Ala<br>Asp-H55 → Ala<br>His-H105 → Ala | 56 ± 2.0 |
| V104 | Asp-L32 → Glu | 33 ± 2.1 |

[a] Activity measurements are normalized to the native Asp-Asp form of the antibody.

IgE significantly, whether the mutant residue was an alanine (V100), glutamate (V104), or the isomerization variants of aspartate (Fab-II and Fab-III). Previous studies (Presta *et al*., 1993) had shown that the affinity of the antibody for IgE can be eliminated by mutating Asp-L30 and Asp-L34 along with Asp-L32, as shown by the result obtained for the V10 mutant. The loss of binding suffered by changing Asp-L32 to Ala can be partially recouped by changing two residues in the heavy chain to Ala, as in the V103 Fab.

## DISCUSSION

Among the modifications undergone by proteins following expression are chemical changes such as the isomerization of an Asp-Gly sequence described here. The biological significance of isomerization of aspartate residues is unknown, but an enzyme has been identified, protein isoaspartyl methyltransferase, that methylates iso-Asp residues and that may be a repair enzyme for reconversion of these residues to Asp (Aswad, 1984; Ota & Clarke, 1989). Hence, isomerization of Asp residues, under certain conditions, likely occurs *in vivo*. The *in vivo* role of isomerization of Asp residues in the CDR of a natural antibody is probably negligible, since polyclonal antibodies consist of a diverse assemblage of sequences with varying affinities, so any secondary chemical effect on a particular sequence will be insignificant. Hence, there would be no evolutionary pressure to avoid labile sequences in the CDR, as there might in other proteins, where significant chemical modifications would have structural and functional consequences for the organism. In the special case of monoclonal recombinant antibodies, which have significant technological importance, chemical modification of CDR residues following expression may be consequential and should be taken into account in the engineering of a therapeutic antibody.

An Asp-Gly sequence in a recombinant human monoclonal antibody was shown here to undergo isomerization to a $\beta$-amino acid, through a cyclic intermediate, giving rise to variants of the antibody that differed at this site, containing either iso-Asp-Gly or succinimide in place of the expected sequence. Identification of the reaction products was facilitated by the use of hydrophobic interaction chromatography for separation of variants of the intact antibody and fragments generated by selective protease digestion. Following digestion with papain, the three variant Fab fragments could be purified by HIC (Figure 4). The residue at the labile position, where Asp was expected, was determined by a

combination of tryptic mapping, mass spectrometry, and N-terminal sequencing. Termination of the Edman sequencing reaction was used to identify the isoAsp residue in a peptide that had the same mass as the expected sequence. Electrospray mass spectrometry of the Fab fragment was sufficiently accurate to demonstrate the increase in mass (18 amu) expected for the cyclic imide-containing variant, even though the fragment itself has a mass near 50 kDa. Pepsin digestion was then employed to generate $F(ab')_2$ fragments, to permit identification of forms of the antibody that carried different combinations of the isomerization product in each of the antigen binding sites. The occurrence of isomerization at a single residue accounted for all of the heterogeneity found in the CDRs of the antibody.

Evidence for isomerization was found in the Asp-L32-Gly-L33 sequence in CDR-L1, but not for the other potential se1uence, Asp-H55-Gly-H56, that is in CDR-H2 of the antibody. The two sequences are both present in loops that interact with antigen and so are exposed to the same environmental conditions during the incubation that gave rise to isomerization in CDR-L1. Analysis by HIC separation, peptide mapping, sequencing, and mass spectrometry indicated only the presence of the expected H2 sequence and no evidence for isomerization at this site. The marked difference in reactivity of the two sequences confirms the role of higher-order structure in the rate of reactions, such as isomerization and deamidation, that proceed in proteins through a cyclic intermediate (Geiger & Clarke, 1987).

Monoclonal antibodies, such as E25, provide a useful tool for investigation of the influence of structure on the reactivity of a peptide sequence since the crystal structures of many Fab fragments have been solved and can be compared to elucidate the likely conformation of a given sequence. In order to investigate why the Asp-Gly sequence in E25 CDR-L1 forms isoaspartate but the same sequence in E25 CDR-H2 does not, we compared CDR loops from Fab crystal structures in which the size and sequence of the CDRs were similar to those in E25. CDR-L1 is a member of the murine $\kappa$-light chain subgroup III, and in this subgroup, a Tyr-Gly sequence is most common, though 8 of 89 sequences contain Asp(or Asn)-Gly (Kabat *et al*., 1991). Only two such crystal structures are available in the Protein Data Bank (Bernstein *et al*., 1977): 1ACY has Tyr-Gly (Ghiara *et al*., 1994) and 1GGB has Asp-Gly (Rini *et al*., 1993). The CDR-L1 loops from these two crystal structures were superimposed using backbone atoms of the first two and last two residues of the CDR (i.e., residues 24, 25, 33, and 34). For these two loops, much of the loop can be superimposed except for the residues near the Tyr-Gly and Asp-Gly sequences (Figure 8, left). For CDR-H2, five Fab crystal structures contain a CDR-H2 with the same size and similar sequence as E25; 1GGB (Rini *et al*., 1993) and 1VFB (Bhat *et al*., 1994) have an Asp-Gly sequence (Asp side chains shown in Figure 8, right), 3HFM (Padlan *et al*., 1989) and 1BAF (Brunger *et al*., 1991) have a Ser-Gly sequence, and 7FAB (Saul & Poljak, 1992) has a Thr-Gly sequence. The five CDRs were superimposed using backbone atoms of residues 49, 50, 59, and 60. In contrast to CDR-L1, the five CDR-H2 loops have similar conformation, with 3HFM being the most dissimilar (Figure 8, right). Four of the five have a type I reverse turn (with the Gly residue as the fourth residue of the turn; 3HFM does not have the turn), and the side chain at position 54 accepts a hydrogen bond from ghe backbone peptide >NH of residue

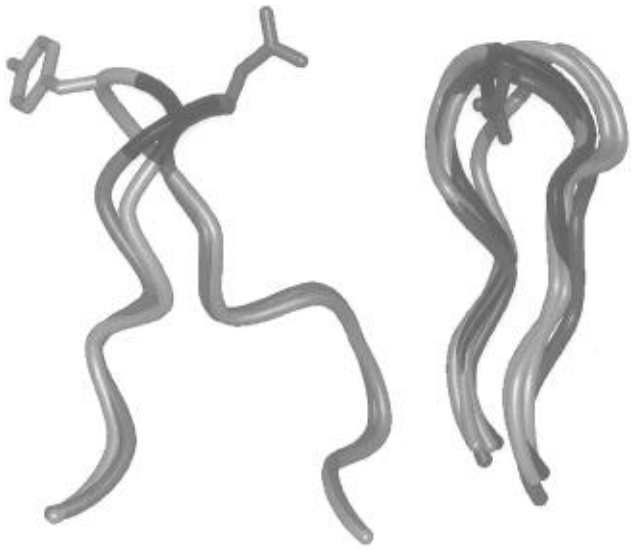

FIGURE 8: Crystal structures of (left) CDR-L1 and (right) CDR-H2 sequences similar to those of E25. The structures of the CDR loops were drawn from published coordinates and superimposed at the first and last two residues of the fragments illustrated as described in the text. The side chains of aspartic acid or asparagine residues in the position corresponding to Asp-L32 or Asp-H55 in the pertinent sequences are shown.

56. The presence of the reverse turn as well as the side chain−backbone hydrogen bonds may stabilize the conformation of CDR-H2 and thereby prevent formation of isoaspartate. The results suggest that the sequence is too constrained to undergo the isomerization reaction observed in CDR-L1. The relatively large differences in crystal structures of the CDR-L1 sequences, on the other hand, suggest that this part of the antibody is relatively unconstrained, accounting for its greater reactivity. Examination of the crystal structures of these sequences thus is a useful tool for the explanation of the large difference in reactivities of the two Asp-Gly sequences in the antibody.

No structural determination of the perturbation caused by isomerization has been reported for a protein, but the potential for alteration in structure is suggested by Figure 1. The products of isomerization differ from the Asp-Gly sequence, with the peptide backbone lengthened by a methylene group in the iso-Asp-Gly variant and incorporating a five-membered ring in the cAsp form. Small changes in sequence of the CDRs have been shown to cause significant losses of binding affinity in E25 (Presta *et al*., 1993), so we examined the effect of isomerization on affinity of binding to IgE. $F(ab')_2$ fragments, which could be purified more easily than variants of intact E25, were tested in an assay for inhibition of binding of IgE to its receptor. The results (Table 3) demonstrate a reduced ability of the products of the isomerization reaction to bind IgE. The forms of E25 that are modified at one antigen binding site retain half the activity of the unmodified $F(ab')_2$ in this assay. Variants that contain isomerization products in both binding sites retain only about 20% of the activity of the fragment with the expected sequence. Hence, isomerization of the Asp-Gly sequence of CDR-L1 of E25 is associated with loss of affinity for antigen.

Further studies incorporating previously reported findings (Presta *et al*., 1993) were undertaken to evaluate modifications to the CDRs that prevent isomerization in the antigen binding sites. A previous study (Presta *et al*., 1993)

demonstrated the key importance of the three Asp residues found in CDR-L1 of E25 on antigen recognition. Mutation of the three residues to alanine eliminates affinity for IgE as shown by the result obtained for the V10 mutant. Mutation of only the labile Asp-L32 residue to alanine reduces the relative potency of the Fab by about 4-fold (Table 4). Substitution of Asp with Ala has the effect of removing the highly polar carboxylate side chain of the acidic residue, without, presumably, altering the protein's α-carbon structure. Hence, this side chain plays a role in affinity for IgE. The resulting mutant cannot undergo isomerization but exhibits an affinity that is only slightly better than that of the variant isoforms (Fab-II and Fab-III) and so is not an improvement over E25. The V104 mutant retains the acidic side chain of residue L32 by substituting a glutamate residue for aspartate, but this mutant also shows significantly reduced affinity for antigen, and also is no improvement over E25. Mutation of His-H105 to alanine has previously been shown (Presta *et al*., 1993) to increase the affinity of E25 for IgE, so this change was incorporated into a mutant in which both potential sites of isomerization were mutated, with a partial improvement in binding affinity, as shown by the result obtained for the V103 mutant. This mutant cannot undergo isomerization, and so represents a chemically stable species, but does not exhibit the same potential as E25 for blocking the binding of IgE to its receptor. These studies demonstrate sensitivity of antigen recognition to subtle sequence changes in the CDRs and the tradeoffs incurred in engineering out a labile sequence.

The work reported here demonstrates the difficulty of predicting whether an Asp residue will undergo isomerization and the need for experimental techniques for making this determination. We have investigated the utility of hydrophobic interaction chromatography (HIC) in the characterization of the antigen binding domains of a monoclonal antibody that binds to IgE. Previous studies have shown that HIC is selective for gross differences in antibody structure and thus has been useful in the purification of bispecific antibodies (Neblock *et al*., 1992), hybrid MAbs (Abe & Inouye, 1993), $F(ab')_2$ fragments (Morimoto & Inouye, 1991), and MAbs containing asymmetric Fab glycosylation (Grebenau *et al*., 1992). Other modes of chromatography, such as chromatofocusing (Jungbauer *et al*., 1990) and cation-exchange (McDonough *et al*., 1989), have been used to identify and purify isoforms found in MAb preparations, generally reflecting much greater sequence and structural variations than expected for a recombinant protein. HIC under the conditions employed here shows high selectivity for the CDR of rhMAbs and therefore may be useful for identification of structural differences that affect antigen binding affinities.

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