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Comparison of the Fc Fragment from a Human IgG1 and Its CH2, pFc′, and tFc′ Subfragments. A Study Using Reductive Methylation and ^{13}C NMR†

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ABSTRACT: The Fc fragment of a human monoclonal IgG1 was compared with subfragments containing (a) the intact CH2 domain (CH2 fragment) or (b) the intact CH3 domain (pFc′ and tFc′ fragments). All fragments were reductively ^{13}C-methylated and their resulting dimethyllysyl resonances characterized in 0.1 M KCl as a function of pH by ^{13}C NMR spectroscopy. Seven resonances were characterized for the 18 lysine residues of the Fc fragment, eight for the 12 lysines of the CH2 fragment, and five each for the 9 lysines of the pFc′ and the 6 lysines of the tFc′ fragments, respectively. The multiplicity of resonances indicates that the lysine residues in each fragment exist in a variety of microenvironments and that the fragments are all highly structured. The correspondence between 6 of the 12 or 13 perturbed lysine residues in the Fc fragment and the smaller subfragments indicates that the conformation of the CH2 and CH3 domains is largely unchanged in the smaller fragments. However, in addition to three lysines at the CH2–CH3 domain interface, whose environments were known to be disrupted in the smaller fragments, three or four lysine residues have somewhat different properties in the Fc fragment and in the subfragments, indicating that some local perturbations are induced in the domain structure in the subfragments. Tentative partial assignments of dimethyllysyl resonances to lysine residues are suggested on the basis of comparisons of the properties of dimethyllysyl resonances in Fc and subfragments and on the interactions of lysyl residues in crystalline Fc [Diesenhofer, J. (1981) *Biochemistry 20*, 2361–2378].

Many of the biological functions associated with the IgG molecule are mediated through macromolecular interactions with sites in the Fc region, which contains the CH2 and CH3 constant domains. These interactions are preserved in proteolytic fragments containing either the Fc region or intact domains. The CH2 domain mediates catabolism of IgG (Dorrington & Painter, 1974) and is associated with complement fixation and antibody-dependent cytotoxicity (Yasmeen et al., 1976; Ellerson et al., 1972), while the CH3 domain has been shown to mediate the heterologous binding of human IgG to guinea pig peritoneal macrophages (Yasmeen et al., 1973), the binding to homologous macrophages (Okafor et al., 1975), and the release of histamine from mast cells (Minta & Painter, 1972).

Because of the biological importance of these immunoglobulin domains, we have embarked on detailed structure–function studies of the Fc fragment and single domain sub-

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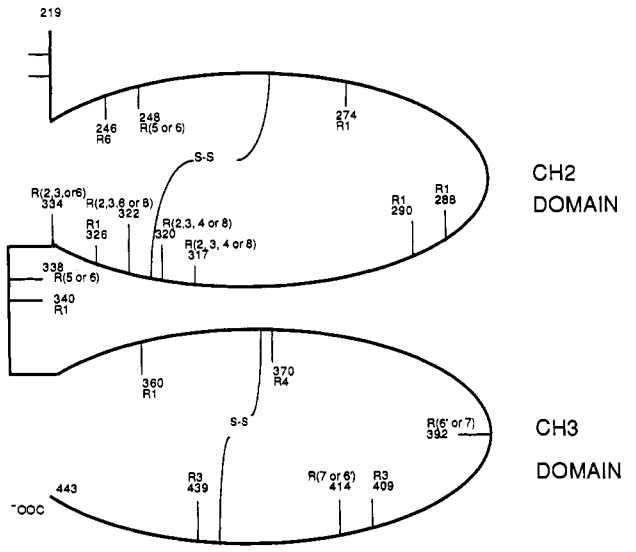

FIGURE 1: The approximate distribution of lysine residues in the polypeptide chain in the CH2 and CH3 domains of the Fc fragment is shown. The lysines are represented by vertical or horizontal lines. The residue number for each lysine is given by the integer next to each line, and the tentative assignment of each lysine to the numbered dimethyllysyl ^{13}C NMR resonances of the Fc fragment is depicted by the number or numbers following "R".

fragments (the CH2 fragment containing the CH_2 domain and the pFc' and tFc' fragments containing the CH3 domain) from a human monoclonal IgG1. Each of the two heavy chain fragments in the Fc fragment contain 18 lysine residues. These residues are found in a variety of microenvironments, and they are reasonably well distributed over both domains, as indicated schematically in Figure 1. The properties of individual lysine residues examined as a group following a change in condition should therefore provide indications of perturbations affecting the domains and, further, indicate whether perturbations are localized or involve the entire fragment. In this report, we compare the Fc, CH2, pFc', and tFc' fragments using reductively ^{13}C-methylated fragments and ^{13}C NMR (Jentoft et al., 1979). The dimethylated lysine residues resulting from the reductive methylation have been shown previously to be sensitive spectroscopic probes of the parent lysine residue (Jentoft et al., 1981; Gerken et al., 1982). Chemical shifts and pK_a values were determined, providing information about the microenvironment of the parent lysine residues. The pFc' and tFc' fragments were compared with each other and with the Fc fragment, and the CH2 domain was compared with the Fc fragment. The similarities and differences in conformation for the lysines in these fragments were deduced, and initial assignments were made of dimethyllysyl resonances to lysine residues.

Materials and Methods

Isolation of IgG-Tu Fragments. IgG-Tu was obtained from the plasma of a patient with gammaopathy as described by Jentoft et al. (1982). The Fc-Tu fragment was obtained by a modification of the method of Porter (Porter, 1959; Jentoft et al., 1982). The last step of the purification of Fc-Tu was absorption to a QAE A-50 anion-exchange column equilibrated with 20 mM triethanolamine, pH 7.6, and elution by the same buffer in the presence of 0.3 M NaCl. The QAE column separated Fc-Tu from contaminating lower molecular weight fragments without resort to low pH. The pFc'-Tu fragment was isolated after pepsin digestion by using the procedure specific for human IgG1 described by Natvig and Turner (1971). The tFc'-Tu fragment was isolated from pFc'-Tu by trypsin treatment (Natvig & Turner, 1971). The CH2 fragment was isolated (in very low yields) after trypsin treatment of IgG-Tu at low pH (Ellerson et al., 1976). All fragments were at least 90% homogeneous, as assessed by SDS–polyacrylamide gel electrophoresis and Ouchterlony double diffusion. Protein concentrations were estimated from absorbance at 280 nm by using values of $OD^{1\%}_{1cm,280nm}$ of 12.3 for Fc-Tu (Jentoft et al., 1982), 15.3 for pFc'-Tu, 16.2 tFc'-Tu, and 12.7 for CH2 (Isenman et al., 1977).

Reductive Methylation. The Fc, pFc', tFc', and CH2 fragments were reductively methylated with [^{13}C]formaldehyde by using the procedure of Jentoft and Dearborn (1979).

^{13}C NMR. All spectra were recorded on a Bruker WH180/270 Fourier transform NMR spectrometer equipped with a Nicolet 1180 computer as described previously (Jentoft et al., 1979, 1981). The sample temperature was typically 26 °C. The free induction decay was accumulated by using 4K or 8K data points with a delay time of 3 s between successive pulses; it was zero filled to 32K data points prior to Fourier transformation. Resolution-enhanced spectra were obtained through Gaussian multiplication of the free inducation decay prior to Fourier transformation.

Protein samples for ^{13}C NMR contained 20% D_2O, 0.1 M KCl, and 0.5% methanol as an internal chemical shift reference at 49.40 relative to TMS. The protein concentrations, except for the CH2 fragment, were 0.2–0.4 mM and required accumulations of 1000–3000 scans per spectrum. The concentration of the CH2 fragment for NMR studies was 0.01 mM, and 7000–15 000 scans were acquired per spectrum.

pH measurements were taken before and after obtaining an NMR spectrum, and the average value, with a range no larger than ±0.03, is reported. The pH meter readings, obtained with a Radiometer PHM pH meter equipped with a combination electrode, were taken as the pH values without correction for the D_2O in the sample. Theoretical titration parameters were fit to each set of pH-dependent chemical shifts by using an iterative program (Dave Starke, personal communication). The chemical shift limits from this program were used to construct a Hill plot for each data set that was then subjected to linear regression analysis in order to obtain the n_H and the goodness of fit. The resulting pK_a values are reported in Table I. The standard deviations of the pK_a values were on the order of ±0.03 pH units, and the Hill coefficient ranged from 0.90 to 1.06 but generally fell within the range of 0.97–1.02 with a typical uncertainty of ±0.03.

The relative areas of the resonances for all the fragments were estimated from integration of the dimethyllysyl resonance, by using spectra that were not resolution enhanced, and assuming that all lysine residues were completely dimethylated. Resonance overlap was an additional cause of uncertainty in the estimation of relative areas.

Results

^{13}C NMR of Methylated Fc-Tu. The ^{13}C NMR spectrum of the ^{13}C-methylated Fe-Tu fragment is shown in Figure 2. The 18 lysine residues of dimethylated Fc-Tu appear as 7 dimethyllysyl resonances. This implies that the lysine residues in the Fc fragment reside in seven distinguishable microenvironments. The ratio of integrated intensities for the dimethyllysyl resonances (43–44 ppm) and the dimethylated N-terminus (around 42 ppm) of a nonenhanced spectrum is 17 to 1, indicating that all the lysines are represented and that the fragment is more than 90% dimethylated. The relative areas of the resonances, normalized to the 18 unique lysines of one chain of the Fc fragment, are shown in Table I. The

Table I: Comparison of the Characteristic Parameters of the Dimethyllysyl Resonances of the Fc, CH2, pFc′, and tFc′ Fragments

fragment	estimated no. of lysines[a]	δ_{HA} (ppm)[b]	$\Delta\delta$ (ppm)[b]	pK_a
		Resonance 1		
Fc[c]	5–6	43.10	1.29	10.13
pFc′	2	43.16	1.09	10.16
tFc′	1	43.10	1.01	10.18
CH2	2	43.02	1.19	10.15
		Resonance 7		
pFc′	3	43.10	1.09	9.83
tFc′	1	43.26	1.05	10.02
CH2	1	43.23	1.28	10.16
		Resonance 2		
Fc	1	42.60	1.06	9.93
CH2	1	43.14	1.01	9.83
		Resonance 3		
Fc	3	43.11	1.16	9.74
pFc′	2	43.19	1.03	9.63
tFc′	2	43.16	0.97	9.68
CH2	2	43.15	0.98	9.74
		Resonance 4		
Fc	3	43.13	1.10	10.50
CH2	1	43.31	1.11	10.67
pFc′	1	43.06	1.09	10.68
tFc′	1	43.08	1.10	10.89
		Resonance 5		
Fc	2–3	43.12	1.43	10.66
		Resonance 6		
Fc	1	43.04	1.49	9.62
CH2	1	43.12	1.17	9.32
		Resonance 6′		
pFc′	1	43.28	1.16	10.04
tFc′	1	43.28	1.16	10.18
CH2	1	42.61	0.79	10.38
		Resonance 8		
Fc	1	42.44	1.54	10.25
CH2	1	42.46	1.05	10.07

[a] Determined by integration of spectra whose FID was modified only by an exponential weighing function of 2 Hz prior to Fourier transform. [b] δ_{HA} is the chemical shift of the fully protonated dimethyllysyl resonance. $\Delta\delta$ is the increase in the chemical shift of the dimethyllysyl resonance upon deprotonation. [c] Resonances 1 and 7 are indistinguishable for the Fc-Tu fragment in 0.1 M KCl. At other salt concentrations, resonance 7 can be seen as a separate resonance with a relative area of one (data not shown).

dimethyllysyl resonances of Fc-Tu were studied as a function of pH in 0.1 M KCl by ^{13}C NMR spectroscopy. Characteristic parameters were derived for each resonance, including chemical shifts, pK_a value, and relative area, as shown in Table I. The value of all the parameters for the dimethyllysyl resonances fall within the range seen for dimethylated lysines in other proteins that have been studied by this method (Gerken et al., 1982; Gerken, 1984). Resonance 1 has titration parameters consistent with a dimethyllysyl residue in a noninteracting, solvent-exposed microenvironment.

^{13}C NMR of Methylated pFc′ and tFc′. The lysine residues in the CH3 domain of IgG-Tu were studied via the dimethylated pFc′ and the tFc′ fragments. The pFc′ fragment, composed of residues 333–443 [using the Eu numbering system (Edelman et al., 1978)], includes the entire CH3 domain with its 6 lysines and a 9-residue, presumably disordered, segment containing 3 lysines from the CH2 domain. The ^{13}C NMR spectra of the dimethylamino region of ^{13}C-methylated pFc′ is shown at several different pH values in Figure 3. There are 5 unique resonances representing its 9 lysines (residues 334, 338, 340, 360, 370, 392, 409, 414, and 439), indicating that these 9 lysine residues are in 5 distinguishable microenvironments. The titration parameters and the estimated number of lysine residues represented by each resonance are given in

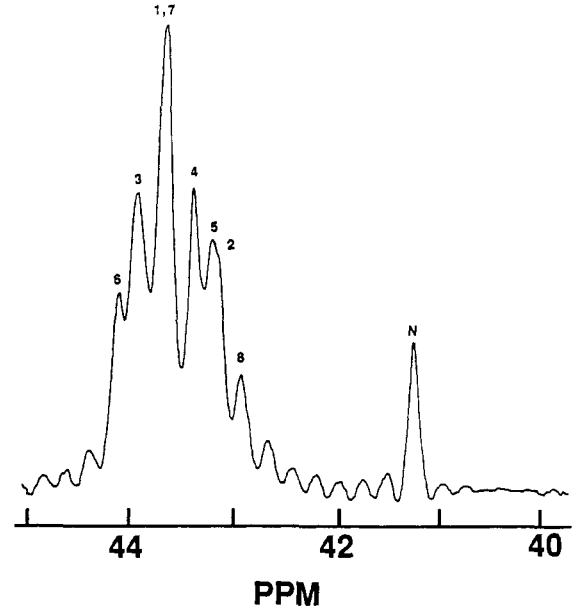

FIGURE 2: ^{13}C NMR spectrum of the dimethylamino resonances from the reductively ^{13}C-methylated Fc-Tu fragment at pH 10.0 in 0.1 M KCl. Conditions for the NMR experiment are given under Materials and Methods. Dimethyllysyl resonances are numbered to correspond to the text. The resonance from the dimethylated N-terminus is labeled N.

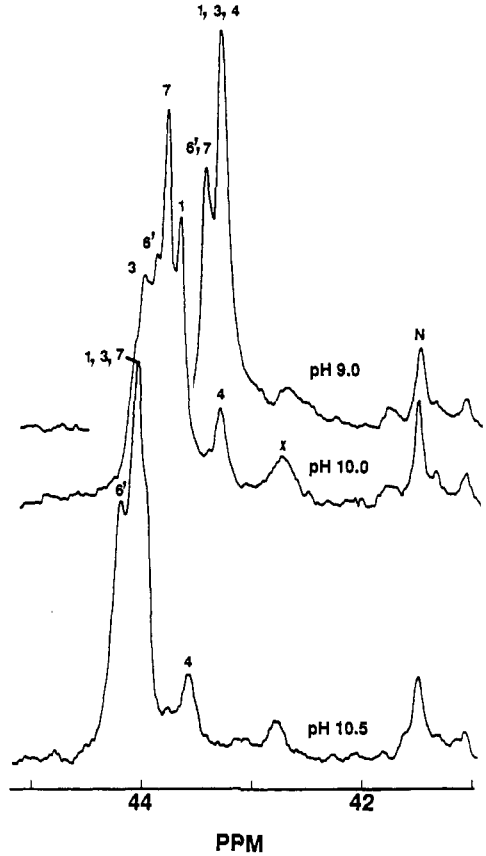

FIGURE 3: ^{13}C NMR spectra of reductively ^{13}C-methylated pFc′-Tu at pH 9.0, 10.0, and 10.5 in 0.1 M KCl. The resonance numbers correspond to the text and are intended to indicate correspondence with the reductively ^{13}C-methylated Fc-Tu resonances. "N" designates the resonance from the dimethylated N-terminus. The broad peak at 41.8 ppm (X) is probably from protein resonances arising from the natural abundance of ^{13}C in the protein.

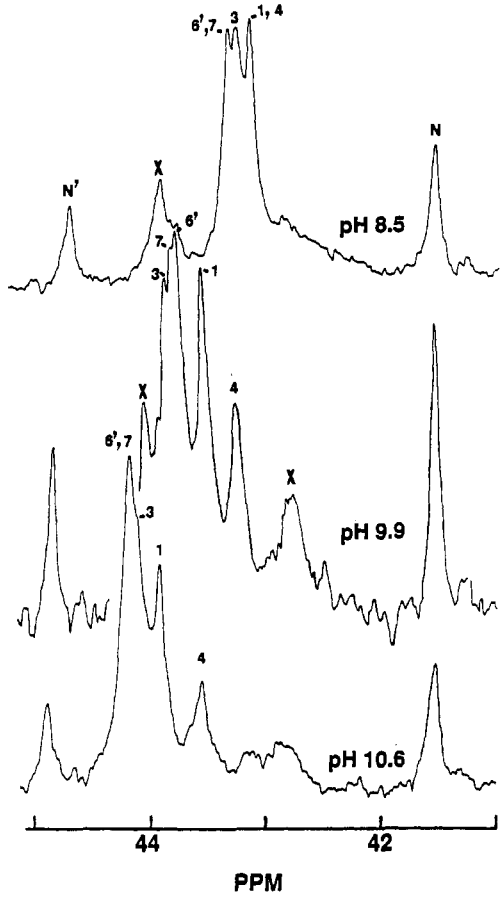

FIGURE 4: ^{13}C NMR spectra of reductively ^{13}C-methylated tFc'-Tu at pH 8.5, 9.9, and 10.6. The resonances are numbered to correspond to the Fc resonances and are discussed in the text. The X's correspond to impurities, probably cyanomethyl adducts which formed as side reactions of the methylation reaction. The resonances labeled N' and N have been assigned to dimethylated N-terminal residues Gly 341 and Ala 339, respectively, on the basis of their titration parameters (data not shown) as discussed in the text.

Table I. Resonance 4, representing a single lysine, has the highest pK_a value, 10.7.

The smaller tFc' fragment, consisting of residues 339 (or 341) to 443 contains, at most, 1 lysine (Lys 340) from the CH2 domain and 6 lysine residues from the CH3 domain. The reductively methylated tFc' fragment was analyzed similarly by ^{13}C NMR spectroscopy. Figure 4 shows the ^{13}C NMR spectrum of reductively methylated tFc' at several pH values. The three dimethyllysyl resonances of approximately equal intensity seen at pH 8.5 separate into 5 distinguishable titrating resonances at higher pH values. Table I lists the titration parameters and the relative areas of the dimethyllysyl resonances of tFc'.

An unexpected feature of this spectrum was the resonance labeled N' at about 45 ppm. This resonance shifts with pH in the range of 6–8 (data not shown) and has a chemical shift consistent with a dimethylated N-terminal glycine residue (Secnik and Jentoft, unpublished observation). Since there are two distinct resonances from dimethylated N-terminal residues (labeled N and N' in Figure 4), the trypsin cleavage of pFc' must occur at two sites, a fact not previously recognized. The tFc' fragment is reported to start at Ala 339 (which presumably gives rise to resonance N) (Natvig & Turner, 1971), at the CH2–CH3 domain interface. The amino acid sequence in the immediate vicinity is Lys_{338}-Ala_{339}-Lys_{340}-Gly_{341}- (Edelman et al., 1969). Our results indicate that about one-third of the trypsin cleavages occur between Lys_{340} and Gly_{341}, while the major cleavage site is between Lys_{338} and Ala_{339}.

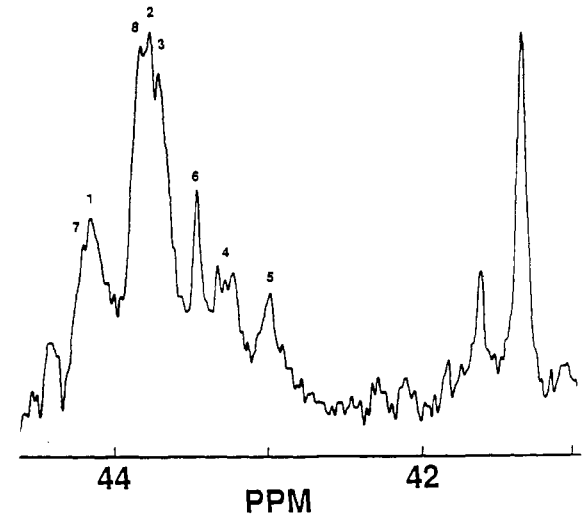

FIGURE 5: ^{13}C NMR spectrum of the dimethylamino resonances of the reductively methylated CH2 fragment from IgG-Tu in 0.065 M KCl, pH 10.05. The resonances are numbered to correspond to the dimethyllysyl resonances of the reductively methylated Fc-Tu fragment, as described in the text. The resonances between 41 and 42 ppm arise from the dimethylated N-termini of the fragment; the smaller peak probably arises from a minor site for H-chain cleavage by trypsin at low pH.

^{13}C NMR of Methylated CH2-Tu. The CH2 fragment consists of one complete CH2 domain (chain A, residues 225–338), the hinge disulfide, and a disulfide-linked fragment of the other CH2 domain (chain B, residues 225–248) (Ellerson et al., 1976). Only chain A contains carbohydrate, attached to Asn 297. There are two lysine residues (Lys 246 and Lys 248) on chain B and 11 on chain A. The ^{13}C NMR spectrum of the reductively methylated CH2 fragment is shown at pH 10.0 in Figure 5. There are 8 well-resolved dimethyllysyl resonances, which give rise to 8 unique titration curves. The titration parameters obtained for the CH2 fragment are shown in Table I. The chemical shift limits and pK_a values were similar to the titration parameters reported above for Fc-Tu, except that there is no CH2 resonance corresponding to resonance 5 of Fc-Tu and no Fc-Tu resonance corresponding to resonance 6' of CH2. Resonance 6' of the CH2 fragment has the lowest pK_a value, 9.32, of all the resonances. Note, however, that the data for the CH2 fragment were obtained in 0.06 M KCl. A closer correspondence between CH2 and the other fragments might have been observed if the CH2 data had been obtained at 0.10 M KCl. Unfortunately, only enough CH2 fragment was available for titration at a single salt concentration.

Discussion

Properties of the Dimethyllysyl Resonances of the Fragments. Seven groups of dimethyllysyl resonances represent the 18 lysyl residues of the Fc fragments, 5 resonances represent the 9 unique lysines of the pFc' fragment, 5 represent the 6 lysines of the tFc' fragment, and 8 resonances represent the 12 lysine residues of the CH2 fragment. The correspondence between the titration parameters of these resonances is indicated in Table I, and the comparison of their titration curves is illustrated in the panels of Figure 6. Resonances from all the fragments have been numbered to indicate their approximate correspondence, using the Fc fragment as the reference molecule.

As indicated previously, resonance 1 has the properties associated with noninteracting, solvent-exposed lysyl residue,

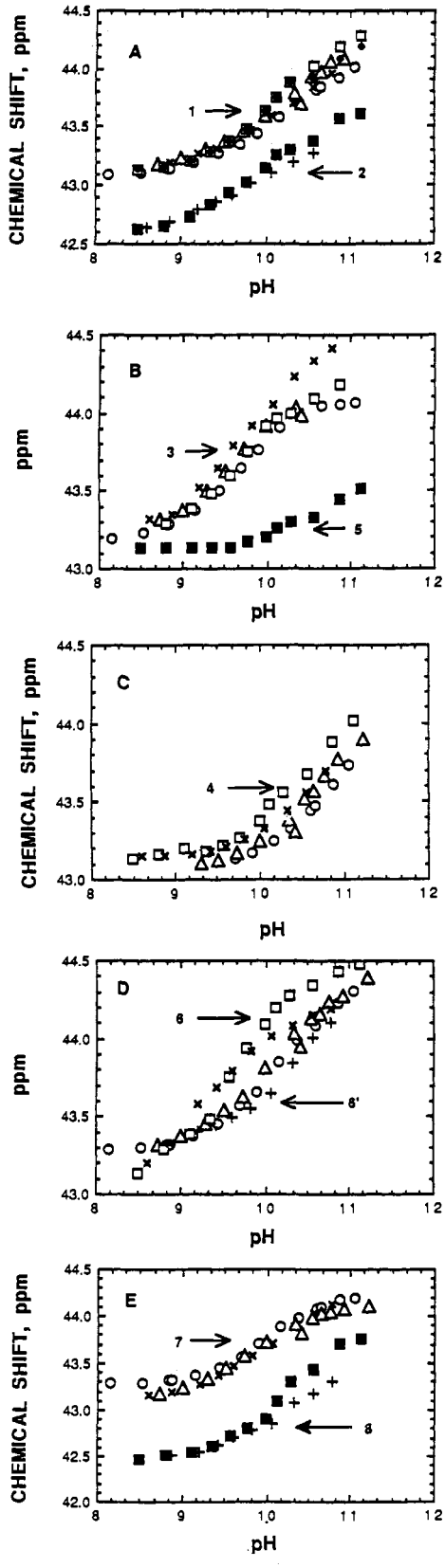

FIGURE 6: Comparison of the titration data for each of the dimethyllysyl resonances of the Fc, CH2, pFc′, and tFc′ fragments. In all panels the fragments are represented by a consistent set of symbols; specifically, the Fc resonances are represented by (□), (■), and (♦) (resonance 7 in panel A only), the CH2 resonances by (×) and (+) the pFc′ resonances by (△), and the tFc′ resonances by (○). The resonances represented in each panel are indicated by the numbers on the arrows and the assignment to resonances is evident, with the exception that in panel D resonance 6 is associated with the Fc resonance (□) and one of the CH2 resonances (×), on the basis of the higher pK_a values of these resonances relative to the 6′ resonances.

namely, a pK_a of 10.2, a δ_{HA} of 43.14 ppm, and a δ_A of 44.14 ppm. Resonance 7 in the Fc fragment is indistinguishable from resonance 1 but is included for completeness since it can be followed as a unique resonance representing a single residue at other salt concentrations (unpublished observation). Resonance 1 (with 7) represents 5 or 6 of the 18-lysine residues in the Fc fragment, while it represents 2 lysines in the CH2 and pFc′ fragments, respectively, and a single residue in the tFc′ fragment. Resonances 5 and 8, representing a total of 5 or 6 residues in the Fc fragment, have pK_a values that are higher than the pK_a value of resonance 1. The parent lysine residues of these resonances are therefore likely to be in microenvironments that stabilize the protonated form of the amino group such as that provided by a hydrogen bond or an ion pair interaction (Gerken et al., 1982). In contrast, resonances 2, 3, and 6, accounting for 5 lysine residues in the Fc fragment, have pK_a values that are less than 10.0. These residues are presumed to be in microenvironments that stabilize the deprotonated form of the amino group, such as that provided by a high local concentration of positive charges or a hydrophobic environment. Lower than normal pK_a values have been observed previously for lysine residues in lysozyme (Gerken et al., 1982) and α-lactalbumin (Gerken, 1984).

In the following section the data summarized in Table I are used to contrast and compare the properties of the dimethylated lysine residues of the fragments containing the CH3 domain, the CH2 domain, and the Fc region which contains both domains. Three classes of dimethyllysyl resonances were identified: (a) those unique to a domain; (b) those unaltered or minimally perturbed and associated with a particular domain; and (c) those altered substantially in the domain fragment relative to the Fc fragment. The pH-dependent properties were also compared with those predicted for specific lysines on the basis of the X-ray structure. The interactions and solvent exposures of the lysine residues in the Fc fragment, derived from the X-ray structure (Deisenhofer, 1981), are summarized in Table II. Tentative or partial assignments of lysine residues to the ^{13}C dimethyllysyl resonances were based both on these comparisons and upon predictions of perturbation based on disruption of CH2–CH3 domain interactions upon producing the single domain fragments.

Comparison of the pFc′ Fragment with the tFc′ Fragment. The CH3 domain encompasses residues 342–443. The pFc′ fragment contains the entire CH3 domain plus 9 additional residues from the carboxy-terminal end of the CH2 domain while the tFc′ fragment contains the entire CH3 domain plus 1 or 3 residues from the CH2 domain. Both the pFc′ and the tFc′ fragments have been shown to retain native secondary (Cathou & Dorrington, 1975) and tertiary structures (Isenman et al., 1977) and biological activity (Yasmeen et al., 1973; Okafor et al., 1975). The isolated CH3 domain exists as a dimer in solution (Turner & Bennich, 1968), and the area of domain–domain interactions is large (1090 $Å^2$ per domain) (Deisenhofer, 1981). Since these CH3–CH3 interactions are preserved in the pFc′ and tFc′ fragments (Turner & Bennich, 1968), residues within the CH3–CH3 contact surface should be in the same microenvironment in Fc, pFc′, and tFc′ fragments. In the CH3 domain, lysines 370, 392, 409, and 439 are involved in intradomain H bonding and/or ion pairing; these should give rise to dimethyllysyl resonances that have very similar titration properties in the Fc fragment and in the tFc′ or pFc′ fragments.

The first nine residues at the N-terminus of the pFc′ or fragment are derived from the CH2 domain and are therefore expected to be disordered. Resonances from lysines in this

Table II: Accessibilities and Interactions of the Lysine Residues of the Fc Fragment[a]

lysine	solvent accessibility[b] (Å²)	site of interaction	type of interaction[c]
		CH2 Domain	
246	47	intra CH2	H bond to NAG 456 OH
248	23	inter CH2–CH3	ion pair with E 380 COO^-
274	44	noninteracting	
288	48	noninteracting	
290	54	noninteracting	
317	41	intra CH2	ion pair with D 280 COO^-
320	26	intra CH2	ion pair with E 333 COO^-
322	35	intra CH2	H bond to Y 278 OH
326	54	noninteracting	
334	31	intra CH2	H bond to V 240 C=O
338	5	inter CH2–CH3	ion pair with E 430 COO^-
340	44	noninteracting	
		CH3 Domain	
360	50	noninteracting	
370	17	inter CH3–CH3	ion pair with E' 357 COO^-
392	33	inter CH3–CH3	H bond to L' 398 C=O
409	0.4	intra CH3	H bond to T 441 OH
		inter CH3–CH3	ion pair with D' 399 COO^-
414	38	intra CH3	H bond to M 358 C=O
439	5	intra CH3	H bond to T 350 OH
		inter CH3–CH3	ion pair with E' 356 COO^-

[a] This information was obtained from the crystal structure data on the Fc fragment (Deisenhofer, 1981). [b] Calculated by using the algorithm of Lee and Richards (1971). [c] Interactions between chains are indicated by a prime after the single-letter code for the second amino acid of the interacting pair.

region (Lys 334, 338, and 340) can be identified by inspection of the relative areas of resonances for the tFc', pFc', and Fc fragments given in Table I. Since Lys 340 is noninteracting in crystalline Fc, we would expect it to be represented by resonance 1 in the Fc and the pFc' fragments. Lysines 334 and 338 form a H bond and an ion pair, respectively, in crystalline Fc, and these interactions are expected to be broken or substantially perturbed in the pFc' fragment (see Table II). Since the relatively unperturbed dimethyllysyl groups represented by resonances 1 and 7 represent three more lysines in the pFc' fragment than in tFc', the dimethyllysyl resonances of Lys 334 and 338 are assigned to resonances 7 in the pFc' fragment (see below for additional discussion). Resonances 3, 4, and 6' from the pFc' and tFc' fragments are similar, indicating that the extra 9 residues at the N-terminus in the pFc' fragment have no effect on the overall structure of the CH3 domain. The tentative assignments of resonances to lysine residues in the CH3 domain is indicated above each residue number in Figure 1.

Comparison of Fc with pFc' and tFc'. Although the CH3 domain retains function, some differences are expected in local structure between pFc' (or tFc') fragments and Fc-Tu because the CH3 domain fragments have lost CH2–CH3 domain interactions. The area of the CH2–CH3 contact surface is 778 Å² (Deisenhofer, 1981), suggesting that, in pFc' and in tFc', the solvent exposure of the CH3 domain will increase by approximately this amount, with resulting changes in the properties of newly exposed residues. For example, the microenvironment of Lys 338 should be different in the Fc and pFc' fragments even if its ion pair with Glu 430 is intact since Lys 338 lies at the CH2–CH3 interface (Deisenhofer, 1981).

Resonances 1 and 3 closely correspond in pFc', tFc', and Fc (see Figure 6, panels A and B). Resonance 1 represents noninteracting solvent-exposed lysines, so similarities among these resonances are expected. Resonance 1 represents a single lysine in tFc', suggesting that it can be assigned to Lys 360, the single noninteracting lysine in the CH3 domain (see Table II). Lysine 340 from the N-terminal "tail" of the pFc' fragment contributes to resonance 1 in this fragment and in the Fc fragment, as discussed above.

Resonance 7 is indistinguishable from resonance 1 in the Fc fragment under the conditions of this experiment. However, in the pFc' and tFc' fragments a distinct resonance 7 exists with an area corresponding to 3 lysines from pFc' and 1 lysine from tFc', respectively. The titration curves for resonance 7 are presented in Figure 6, panel E. The pK_a value of resonance 7 for each of these fragments is similar to that of resonance 1 (see Table I), suggesting that resonance 7 represents lysine residues at sites in the pFc' and tFc' fragments where the effective dielectric is close to that of water. However, since the chemical shift is perturbed from that of resonance 1, the parent lysines for this resonance may be near aromatic residues that generate ring current effects. By comparing the pFc' and tFc' fragments, we conclude that the three lysine residues from the CH2 domain in the pFc' fragment (Lys 334, 338, and 340) give rise to half of the area in resonance 1 (Lys 340) and two-thirds of the area in resonance 7 (Lys 334 and 338). These lysines are certain to be in nonnative environments in the pFc' and tFc' fragments since they are derived from the CH2 domain, but the lysines in resonance 7 may still lie in a partially structured environment. The remaining residue contributing to resonance 7 in the pFc' and tFc' fragments could be either Lys 392 or Lys 414 since each is highly exposed to solvent, and each is potentially close enough to an aromatic residue to have its chemical shift perturbed by ring currents. Specifically, in crystalline Fc, Lys 392 is within 11 Å of Tyr 391 and Lys 414 is within 11 Å of Trp 417.

Resonance 3 has essentially the same properties in the pFc', tFc', and Fc fragments, suggesting that the environment of its parent lysine residues is the same in the isolated CH3 domain and in the larger Fc fragment. Therefore, we suggest that resonance 3 in all three fragments arises from the buried, interacting lysines in the CH3 domain, Lys 409 and 439, each of which forms an ion pair across the stable CH3–CH3 domain interface as well as a hydrogen bond within its domain (Table II). One additional residue, presumably in the CH2 domain, also contributes to resonance 3 in the Fc fragment (Table I). The titration curves for resonance 3 from the various fragments are compared in Figure 6, panel B.

Lysines 409 and 439 belong to a special category of lysine residues because their amino groups form the maximum number of interactions. In crystalline Fc their amino groups are aligned to form three hydrogen bonds, two in the ion pair interaction with a carboxyl group and one in a hydrogen bond to a threonine hydroxyl group (see Table II). A special situation exists upon dimethylation for lysines that form more than one hydrogen bond, because the dimethylated amino group can only form a single hydrogen bond. Thus, dimethylation of Lys 409 and Lys 439 will probably perturb these residues in *all* of the fragments. Note that dimethylation does not alter the charge on the amino group, so both Lys 409 and Lys 439 in their dimethylated form could still form ion pairs, so long as the ion pairs do not require stabilization by hydrogen bonds. We cannot tell what situation exists for these residues in our fragments, but we would expect Lys 409 and Lys 439 to be in the same environment in the Fc, pFc', and tFc' fragments, consistent with their assignment to resonance 3.

Resonance 4 in the pFc' and tFc' fragments represents a single lysine with similar pK_a values to resonance 4 in the Fc fragment (Table I and Figure 6, panel C). Resonance 4 has a higher than normal pK_a value, suggesting that its parent

lysine may be involved in a strong ion pair interaction. Lysine 370 is a good candidate, as it forms an ion pair across the CH3–CH3 interface (Table II) and is only about 30% solvent exposed. The difference in protonated chemical shifts between Fc and tFc′ or pFc′ fragments suggests that a small change occurred in the groups surrounding Lys 370 upon generation of the single domain fragments.

Resonance 6′ in the pFc′ and tFc′ subfragments has different properties than resonance 6 in the Fc fragment (Figure 6, panel D). In fact, the pK_a values of resonance 6′ are very similar to those of resonance 1 (Table I), but it is distinguished by the unusual value of 43.26 ppm for the chemical shift of the fully protonated form. Residues that are good candidates for this resonance are Lys 392 and Lys 414 (Table II); one of these residues is probably associated with resonance 7 and one with resonance 6′. Although we cannot distinguish between these residues at this time, we note that the residue associated with resonance 6′ exists in an environment that is perturbed by disruption of the CH2–CH3 interface.

Comparison of the CH2 and Fc Fragments. The structure of the CH2 fragment is less well-defined than those of the CH3 domain fragments because the consequences of disrupting potential carbohydrate–carbohydrate interactions between the two CH2 domains are unknown as are the structural consequences of retaining the disulfide-linked polypeptide remnant of the second heavy chain. Nevertheless, this fragment is the only characterized fragment for human IgG that contains an intact, isolated CH2 domain.

Resonance 1 from CH2 arises from 2 lysines and correlates well to resonance 1 of Fc (Figure 6, panel A, and Table I). In addition, resonance 7 from CH2 arises from a single lysine (Table I), so that a total of 3 residues in the CH2 domain appear to be noninteracting. Six noninteracting residues were expected from the X-ray structure (Table II), namely, Lys 274 (A and B chains), Lys 288 (A and B chains), Lys 290, and Lys 326. Thus, unexpectedly, the lysines in the CH2 domain appear to be more structured in the CH2 fragment in solution than in soluble or crystalline Fc fragments.

Resonance 2 from Fc and CH2 correlates quite well (Table I and Figure 6, panel A). This resonance represents a single lysine in each fragment and is thus unique to the CH2 domain. The environment of the parent residue is not affected by fragmentation. Resonance 3 of CH2 agrees reasonably well with resonance 3 of the Fc fragment (Figure 6, panel B). In the dimethylated Fc fragment, 1 or 2 of the 3 lysines of resonance 3 apparently derive from the CH2 domain (Table I). Resonances 2 and 3 have depressed pK_a values (Table I) that could arise as a result of solvent-protected ion pair interactions or of some other environment of decreased effective dielectric. Candidates for resonances 2 and 3 are the same, namely, lysines 317, 320, 322, and 334 (Table II).

Resonance 4 is quite similar for the CH2 and the Fc fragments (Table I and Figure 6, panel C). The pK_a for this resonance is elevated, as would be expected for a lysine in an ion pair interaction. Lysines 317 and 320 are therefore the best candidates for this residue in the CH2 domain (Table II).

Resonance 5 of Fc, which represents 2 or 3 lysine residues (Table I), does not correlate with any CH2 or tFc′ resonances, primarily because none of the resonances in these fragments undergo such a large change in chemical shift upon titration (see Figure 6, panel B). Resonance 5 of the Fc fragment could arise from lysines in the CH2 domain in ion pair interactions, since this resonance has a higher than normal pK_a value (Table I). Lysines 248 and 338, which form ion pairs across the CH2–CH3 interface (Table II) and which are close to aromatic residues, are candidates for this resonance.

Resonance 6 of Fc, which represents a single lysine (Table I), does not correlate with any subfragment resonance, primarily because, like resonance 5, it undergoes a large chemical shift change upon titration (Table I and Figure 6, panel D). The pK_a value of this resonance is lower than normal, which could reflect either an environment of decreased effective dielectric or a solvent-protected ion pair. Lysine residues that are also close to aromatic residues at the CH2–CH3 interface of the Fc fragment are candidates for this resonance, since the local environment of either the lysine and/or the aromatic residue could change upon formation of subfragments. Candidates include lysines 246, 248, 334, and 338.

Resonance 6′ exists in the CH2, pFc′, and tFc′ fragments (Table I and Figure 6, panel D), suggesting that in each domain there is a resonance whose microenvironment is similarly altered from its state in the Fc fragment to a new microenvironment. In the CH3 domain, this resonance was tentatively assigned (see above) to Lys 392 or 414 (Table II). By analogy, Lys 246, 322, or 334 could be candidates from the CH2 domain for assignment to resonance 6′. Lysine 246 is an interesting possibility since its hydrogen bond to the *N*-acetylglucosaminyl moiety (NAG) of the carbohydrate chain (Table II) is likely to be perturbed in the single domain CH2 fragment.

Resonance 8 of CH2 represents a single lysine that is not correlated to any Fc resonance (Table I). It resembles resonance 8 of the Fc fragment only in its unusual chemical shift in the fully protonated state (see Figure 6, panel E), but its pK_a value in the CH2 fragment is closer to that of a noninteracting lysine residue (Table I). Candidates for this resonance in the Fc and CH2 fragments are Lys 319, 320, and 322, which are near to aromatic residues in crystalline Fc (Deisenhofer, 1981).

Five of the CH2 dimethyllysyl resonances correspond reasonably well to resonances from the Fc fragment, suggesting that a substantial region of the CH2 domain is unaffected when the CH2 domain fragment is generated. Several lysine residues in this fragment formed interactions across the CH2–CH3 interface in the Fc fragment, presumably giving rise to resonances 5, 6′, and 8 in the CH2 fragment. Lys 246, 248, and 338 are almost certain to contribute to these CH2 resonances. These tentative assignments of resonances to lysine residues are summarized in Figure 1.

Conclusion. This work illustrates the power of incorporating multiple amino acid specific ^{13}C NMR probes onto a large macromolecule and shows that such probes can be used to elicit both local and global information about the system. Specifically, this study demonstrates the similarities and differences in the local structure of the CH2 and CH3 domains of human IgG1 when these domains are intact within the Fc fragment and when they are largely isolated in subfragments. The data are consistent with the fragments generally maintaining the interactions and microenvironments found for lysine residues in crystalline Fc (Diesenhofer, 1981), suggesting that there is a close correspondence between the structure in solution and that in the crystal for the human Fc fragment. Prior to this study, detailed information regarding local microenvironments on these fragments in solution was not available.

By comparing the parameters for the dimethyllysyl resonances of Fc, pFc′, and tFc′, we have made tentative assignments of all the lysines residues in the CH3 domain to the ^{13}C NMR resonances of the reductively methylated pFc′ and tFc′ fragments. Several of the resonances from the reductively methylated CH2 fragment have also been tentatively asso-

ciated with lysine residues. By comparing the ^{13}C NMR data from the reductively methylated CH2 fragment, the reductively methylated pFc′ and tFc′ fragments, and the reductively methylated Fc fragment, we were able to assign dimethyllysyl resonances in the Fc fragment to domains. For example, resonances 2 and 8 each represent single lysine residues in the CH2 domain, and we have suggested candidate lysine residues for each of these resonances.

The assignments will be extended and verified in additional studies of this system. For example, we plan to use the salt dependence of the dimethyllysyl resonances to confirm the identity of lysyl resonances in ion pair interactions. An ultimate goal of these studies is to use the dimethylated Fc fragment to study its interactions with effector molecules such as C1q and the Fc cell surface receptor.

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Mitochondrial NADH–Ubiquinone Reductase: Complementary DNA Sequences of Import Precursors of the Bovine and Human 24-kDa Subunit

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ABSTRACT: The 24-kDa subunit of mitochondrial NADH–ubiquinone reductase (complex I) is an iron–sulfur protein that is present in the flavoprotein or NADH dehydrogenase II subcomplex. It is a nuclear gene product and is imported into the organelle. A group of human patients with mitochondrial myopathy have been shown to have reduced levels of subunits of complex I in skeletal muscle mitochondria, and in one patient the 24-kDa subunit appears to be absent (Schapira et al., 1988). To investigate the genetic basis of this type of myopathy, cDNA clones have been isolated from a bovine library derived from heart and liver mRNA by hybridization with two mixtures of 48 synthetic oligonucleotides 17 bases in length that were designed on the basis of known protein sequences. The recombinant DNA sequence has been determined, and it encodes a precursor of the mature 24-kDa protein. The N terminus of the mature protein is preceded by a presequence of 32 amino acids that has properties that are characteristic of mitochondrial import sequences. The sequence of the mature protein deduced from the cDNA contains a segment of nine amino acids that was not determined in an earlier partial protein sequence analysis. The bovine clone has been employed as a hybridization probe to identify cDNA clones of the human homologue of the 24-kDa protein. Its DNA sequence has also been determined, and it codes for a protein that is closely related to the bovine protein. Three conservative substitutions are found in the mature protein, and the human and bovine presequences differ at a further five positions. The bovine cDNA clone has been used as a hybridization probe with digests of bovine and human DNA to investigate the genetic complexity of the protein.

Mitochondrial NADH–ubiquinone reductase (complex I) is embedded in the inner membrane of the organelle. It is the first enzyme of the respiratory chain and catalyzes the reoxidation of NADH and transfer of electrons to ubiquinone [for a review see Ragan (1987)]. For each electron transferred between NADH and ubiquinone two protons are pumped from the mitochondrial matrix (Wikström, 1984). Complex I is by

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