Photo-CIDNP Studies of Bence Jones Proteins, Immunoglobulins, and Their Proteolytic Fragments

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ABSTRACT: The results of photo-CIDNP measurements of Bence Jones proteins, immunoglobulins, and their proteolytic fragments are reported. The CIDNP spectra of constant region C_L fragments, which were derived from λ-type Bence Jones proteins with three different isotypes, gave polarizations originating from two Tyr and one Trp residues. From comparisons of the results obtained by using λ-type Bence Jones proteins that possess the known amino acid sequences with those for the C_L fragments, it was concluded that the CIDNP signals observed in the case of Bence Jones proteins are all due to the hypervariable region. On the basis of the results obtained by using IgG1, IgG2, IgG3, and IgG4 myeloma proteins along with their Fab, Fc, and pFc′ fragments, it was concluded that CIDNP signals for the Fab region all originate from the variable region. The Fc region gives two Tyr polarizations, one of which has been assigned to Tyr-296. We have also shown that the second Tyr signal is due to Tyr-373. Interaction of Fc and staphylococcal protein A has also been examined by the CIDNP technique.

Bence Jones proteins, which are excreted into the urine of patients with multiple myeloma, are dimers of homogeneous light chains of immunoglobulins. The light chains are divided into C_L and V_L, which are two homology units of about 110 amino acid residues.[1] Light chains can be of either λ or κ type. Proteins of the human immunoglobulin G (IgG) class can be differentiated into four subclasses (IgG1–IgG4), each with a distinctive heavy chain, γ1, γ2, γ3, or γ4. The γ chains consist of four homology units, V_H, C_H1, C_H2, and C_H3. The variable domains V_L and V_H differ markedly from one protein to another, whereas the constant domain C_L, C_H1, C_H2, and C_H3 have essentially invariant sequences.[2]

The photo-CIDNP technique can give information about the accessibility of His, Tyr, and Trp residues on the surface of proteins to photoexcited flavin dyes (Kaptein et al., 1978a,b; Kaptein, 1982). In the present paper we report the results of analyses of CIDNP spectra obtained by using λ-type Bence Jones proteins, intact IgG, and their proteolytic fragments. We will discuss how aromatic residues that exist in the variable region of Bence Jones proteins and IgG are responsible for the observed CIDNP signals. Assignments of Tyr signals originating from the Fc region will also be discussed.

Materials and Methods

All protein preparations used in the present experiment were isolated and purified as described previously (Arata & Shimizu, 1979; Shimizu et al., 1980, 1983; Arata et al., 1980; Endo & Arata, 1985). A total of 1–5 mg of C_L fragments and 2–10 mg of the Bence Jones proteins, Fab fragments, Fc fragments, and intact IgG proteins were dissolved in 0.3 mL of 0.2 M $NaCl/D_2O$. The pH was adjusted with 0.3–0.5 M DCl and NaOD. All pH values reported in this paper are uncorrected meter readings of D_2O solutions made with an electrode standardized by using H_2O buffers. A flavin dye, 3-(carboxymethyl)lumiflavin, was added to the sample solutions at a concentration of about 0.2 mM. NMR measurements were performed at 30 °C.

Table I: Amino Acid Substitutions for Three Kinds of Isotypes of the Human λ Chain

	position[a]				
isotype	113	115	153	164	190
Mcg(−), Oz(−)	Ala	Ser	Ser	Thr	Arg
Mcg(−), Oz(+)	Ala	Ser	Ser	Thr	Lys
Mcg(+), Oz(−)	Asn	Thr	Gly	Lys	Arg

[a] The numbering system is based on the protein Sh [see Putnam et al. (1967)].

^1H NMR spectra were obtained at 500 MHz on a JEOL GX-500S NMR spectrometer. A probe head with a 3-mm quartz rod was used for the photo-CIDNP measurements. The instrumental setup was basically the same as designed and reported by Kaptein et al. (1978a). We used an NEC GLG-3300 argon ion laser as the light source. Prior to the measurements, laser irradiation time, laser power, number of accumulations, and the delay time have been checked for the optimum experimental condition. Irradiation times of 50–100 ms were used to avoid the effect of cross polarization (Hore et al., 1982). A presaturation pulse train with pulse intervals divided step by step by a factor 2, i.e., $\pi/2$–800 ms–$\pi/2$–400 ms...$\pi/2$–12 ms, was applied prior to the laser pulse. Alternating light and dark free induction decays with a pulse delay time of 6 s were collected. Typically 50–100 free induction decays were accumulated. No significant flavin bleaching

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[1] Abbreviations: CIDNP, chemically induced dynamic nuclear polarization; C_L, the constant fragment of the light chain; DSS, 4,4-dimethyl-4-silapentane-1-sulfonic acid sodium salt; Fab, antigen binding fragment; Fc, fragment composed of the C-terminal halves of the heavy chains; IgG, immunoglobulin G; NMR, nuclear magnetic resonance.

[2] The nomenclature for immunoglobulin G and its fragments is as recommended in *Bull. W.H.O.* (1964).

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Table II: Amino Acid Sequences of the V_L Domains of the Four Bence Jones Proteins Used in the Present Experiment[a]

	28	29	30	33	34	37	47
Ak	Arg	Asn	Phe	Trp	Tyr	Leu	Tyr
Ma	Asp	Asn	Thr	Trp	Tyr	Val	Tyr
Sh	Gly	Tyr	Asp	Trp	Tyr	Lys	Tyr
Mcg	Tyr	Asn	Tyr	Trp	Tyr	His	Tyr

	52	67	77	84	85	89	95
Ak	Arg	Thr	Gln	Tyr	Tyr	Trp	Val
Ma	Trp	Thr	His	Tyr	Phe	Trp	Gly
Sh	Arg	His	Gln	Tyr	Tyr	Arg	His
Mcg	Arg	Asn	Gln	Tyr	Tyr	Tyr	Phe

[a] Only the relevant parts with His, Tyr, and Trp residues are given. The aromatic residues that exist in the core region of the V_L domain are indicated by boxes. Underlines indicate that the residues are located in the hypervariable region.

occurred under the present experimental condition. The subtraction of the dark and light spectra yielded a photo-CIDNP difference spectrum.

Results and Discussion

Bence Jones Proteins. λ-type Bence Jones proteins Ak, Ma, Sh, and Mcg with known amino acid sequences (Kametani et al., 1983; Takahashi et al., 1980; Titani et al., 1970; Fett & Deutsch, 1974) were used in the present experiment. Proteins Ak, Ma, and Sh are of Mcg(−), Oz(−) isotype, whereas protein Mcg belongs to Mcg(+), Oz(−) isotype. Isotypic substitutions for the proteins used in the present work are summarized in Table I.[3] Proteins with the identical isotype would have variations of amino acid residues only in the variable region V_L. Table II summarizes the amino acid variations for the four proteins used in the present experiment. Photo-CIDNP spectra observed for all of the Bence Jones proteins are compared in Figure 1. It is noted that chemical shifts observed for the four λ-type Bence Jones proteins are all significantly different from each other.[4] No signals possess identical chemical shifts. These results strongly suggest that all of the CIDNP signals observed for the four λ-type Bence Jones proteins originate from the hypervariable region.

Sh and Mcg proteins, which give strong Tyr polarizations in the aromatic region, do not give any detectable signals in the aliphatic region. We have frequently encountered difficulties of detecting aliphatic photo-CIDNP resonances in proteins with the size that is comparable to or larger than that of Bence Jones proteins. An increase in the size of proteins would generally result in a significant degree of contribution from spin diffusion of polarization. The aromatic rings of His, Trp, and Tyr residues that give CIDNP signals presumably are exposed to solvent and can have an additional degree of freedom of motion with respect to the C_β–$C\gamma$ bond. It is

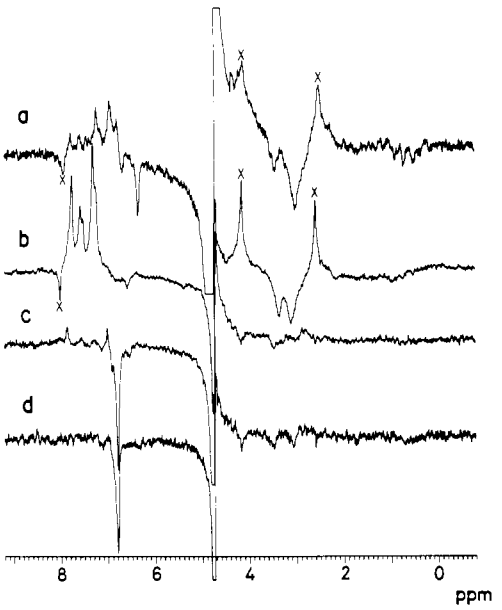

FIGURE 1: Photo-CIDNP difference spectra of four λ-type Bence Jones proteins: (a) protein Ak; (b) protein Ma; (c) protein Sh; (d) protein Mcg. Proteins Ak, Ma, and Sh belong to the same isotype Mcg(−), Oz(−), whereas protein Mcg belongs to the Mcg(+), Oz(−) isotype. The polarizations marked × are due to the flavin dye. Ten milligrams of Ak, Ma, and Sh and 4.2 mg of Mcg were dissolved in 0.3 mL of 0.2 M $NaCl/D_2O$. pH: (a) 6.82; (b) 7.18; (c) 7.00; (d) 6.71. One hundred scans were accumulated with a laser irradiation time of 100 ms.

therefore likely that the aromatic proton signals are less susceptible to the effect of spin diffusion than the C_β–H proton signals. In addition, the mechanisms for the generation of CIDNP signals are different, which is governed by hyperconjugation and exchange interaction for the aliphatic and aromatic protons, respectively. These may be the reasons for the apparent failure of observing the aliphatic resonances.

It is observed in Figure 1 that Bence Jones protein Ak gives Tyr and Trp polarizations. Inspection of Table II indicates that these polarizations are due to Tyr-47 and Trp-89.[3] Bence Jones protein Ma is observed to give a set of strongly polarized signals from a Trp residue. Protein Ma possesses two Trp residues, i.e., Trp-52 and Trp-89. Although we cannot assign the polarized Trp to one of these two Trp residues, it is certain that the CIDNP signal does come from one of the Trp residues that exist in the hypervariable region.

In the case of protein Sh, two Tyr and one His residues are observed to be polarized. The Tyr polarizations showed large pH dependence around neutral pH. This suggests that Tyr residues have His neighbors. The two polarized Tyr residues can be assigned to Tyr-29 and Tyr-47, which exist in the hypervariable region. One of the two His residues, His-67 and His-95, that exist in the hypervariable region must be responsible for the polarized His residue for protein Sh. It is observed that in protein Mcg more than one Tyr residue with similar chemical shifts appear to be polarized. Presumably these Tyr residues are also located in the hypervariable region.

According to the X-ray crystallographic data obtained for Bence Jones protein Mcg (Edmundson et al., 1975), which is actually one of the four proteins used in the present measurements, several hypervariable loops comprise the antigen binding site that is constructed on the β barrel (Novotny et al., 1983; Novotny & Haber, 1985). The results obtained above for the λ-type Bence Jones proteins are summarized in Figure 2, where the folding of the backbone of the polypeptide chain is shown on the basis of the X-ray crystallographic data reported for protein Mcg (Edmundson et al., 1975). It is of

[3] The numbering system used in the present paper is based on protein Sh for the λ chain (Putman, 1969) and on protein Eu for the γ1 chain (Edelman et al., 1969).

[4] The Tyr chemical shifts observed for the CIDNP resonances for Sh and Mcg proteins are apparently close to those for denatured proteins. Sh and Mcg proteins are among those investigated extensively in our previous NMR work (Arata & Shimizu, 1979). It was confirmed by taking NMR spectra that Sh and Mcg proteins used in the present study are in the native state.

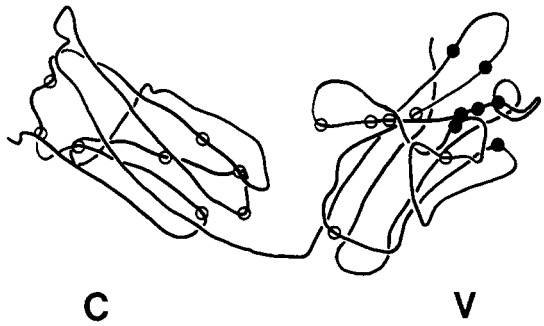

FIGURE 2: Distribution of His, Tyr, and Trp residues giving CIDNP signals, based on the X-ray crystallographic data reported for Bence Jones protein Mcg (Edmundson et al., 1975). Open and solid circles indicate the positions of His, Tyr, and Trp residues included in the four Bence Jones proteins used in the present experiment. The solid and open circles show the residues that do and do not give rise to CIDNP polarization, respectively.

great interest that all the residues that give photo-CIDNP signals exist in the hypervariable loops, which are presumably responsible for the antigen recognition. It appears that on the surface of the Bence Jones protein molecule the antigen binding site is the only region where *CIDNP active* His, Tyr, or Trp residues exist. It has been shown that the necessary condition for these aromatic residues giving photo-CIDNP signals is that His and Tyr residues possess the freely accessible imidazole NH and phenol OH groups, respectively, and the Trp residue possesses the exposed indole ring (Kaptein, 1982). This result suggests the importance of the photo-CIDNP technique in studying the antigen–antibody interaction.

Photo-CIDNP spectra of C_L fragments derived from six kinds of λ-type Bence Jones proteins with the known isotypes are shown in Figure 3. The C_L fragment of the λ-chain has two His, four Tyr, and two Trp residues. In each spectrum resonances designated Tyr 1, Tyr 2, and Trp are observed. We have checked the effect of cross polarization by changing the laser irradiation time. For example, spectrum f was observed by using a laser irradiation time of 100 ms, whereas a laser irradiation time of 50 ms was used for the observation of the rest of the spectra. No significant difference in intensity exists for these spectra. As shown later in the case of much larger fragments such as Fab, Fc, and pFc′, no significant contribution has been observed from cross polarization when irradiation times of 50–100 ms were used. We therefore conclude that both Tyr 1 and Tyr 2 originate from the directly polarized 3,5-H protons of two different Tyr residues. We apparently fail to observe aliphatic resonances corresponding to both of the two Tyr residues, probably due to a poor signal-to-noise ratio for Tyr 2. Signals marked × in the aromatic region do not have in the dark spectra any counterpart with intensity comparable to that for Tyr 1 and Tyr 2, indicating that it is due to a contaminant. In view of the fact that signal × is observed in all of the C_L fragments examined, we conclude that it is due to a byproduct of the limited digestion of Bence Jones proteins. Among possible candidates are a Tyr-containing peptide and a modified C_L fragment with proteolytic cleavage site(s) introduced in the neighborhood of one of Tyr residues.

As Figure 3 shows, each C_L spectrum gives a similar pattern. However, small changes in chemical shift are observed for the light chains with different isotypes. The isotypic substitutions for amino acid residues of the λ chains are summarized in Table I. Since no Tyr or Trp residues are included in the isotypic substitutions, spectral assignments for the polarized signals cannot be made by comparing each of the observed spectra. In a previous paper (Shimizu et al., 1980) it has been

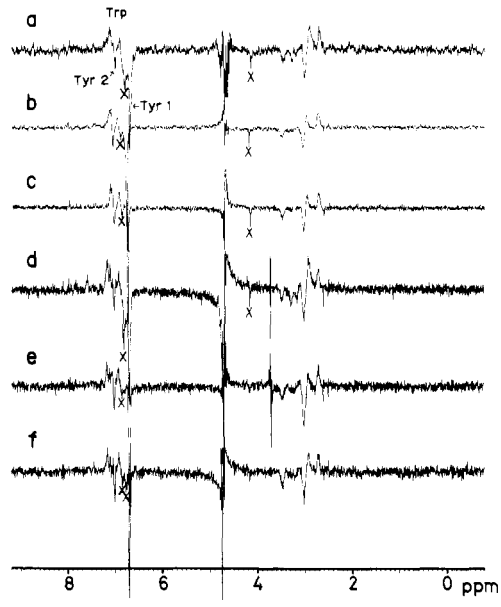

FIGURE 3: Photo-CIDNP difference spectra of six C_L fragments of the λ-type Bence Jones protein: (a) protein Nag; (b) protein Uts; (c) protein Kob; (d) protein Hiro; (e) protein Jon; (f) protein Ma. Isotypes of these proteins are (d–f) Mcg(−), Oz(−); (b and c) Mcg(+), Oz(−); and (a) Mcg(−), Oz(+). Signals marked × are due to either the flavin dye or contaminants. Experimental conditions are as follows: (f) 64 scans, 100-ms laser irradiation time; (a–e) 40 scans, 50-ms laser irradiation time. For (a) 1.2 mg, (b) 1.7 mg, (c) 2.0 mg, (d) 2.1 mg, (e) 2.2 mg, and (f) 2.5 mg were dissolved in 0.3 mL of 0.2 M $NaCl/D_2O$. pH: (a) 7.30; (b) 7.13; (c) 7.21; (d) 7.20; (e) 7.62; (f) 7.91.

shown that changes in chemical shifts observed for the λ chains with different isotypes are quite consistent with X-ray crystallographic data and suggested that the isotypic substitutions, which exist only in the loop region, do not significantly affect the backbone structure of the immunoglobulin fold. The X-ray crystallographic data (Edmundson et al., 1975) indicate that Tyr-140 and Tyr-173 are in close spatial proximity with position 164, where an amino acid substitution from Thr to Lys occurs for Mcg(+) proteins. Comparisons of photo-CIDNP results observed for Mcg(+) proteins (spectra b and c) and Mcg(−) proteins (spectra a, d–f) clearly show that two Tyr residues give significant shifts for the two kinds of proteins. This suggests that the Tyr residues that give photo-CIDNP signals are Tyr-140 and Tyr-173. The C_L domain of the λ chain possesses two Trp residues, i.e., Trp-148 and Trp-186. The X-ray crystallographic data show that Trp-148 is buried in the interior of the immunoglobulin fold, whereas Trp-186 is exposed to solvent. This indicates that the Trp polarization originates from Trp-186. It should be noted that a significant deviation of chemical shift is observed for the Trp signal for the Mcg(+) and Mcg(−) proteins. We therefore suggest that, consistent with our previous paper (Shimizu et al., 1980), Mcg(+) and Mcg(−) proteins have slightly different conformation in solution.

It should be noted that the Bence Jones proteins examined in the present work do not give any polarizations originating from Tyr-140, Tyr-173, and Trp-186. Failure to observe the Tyr polarizations is quite consistent with the results of the X-ray crystallographic study (Edmundson et al., 1975), which has demonstrated that Tyr-140 and Tyr-173 are located in the junction of the V_L and C_L domains. By contrast, Trp-186 is exposed to solvent (Edmundson et al., 1975; Kawata et al., 1988). The C_L fragment is known to exist as the monomer in solution (Karlsson et al., 1972). An increase in the size of the molecules from the C_L fragment to the Bence Jones protein

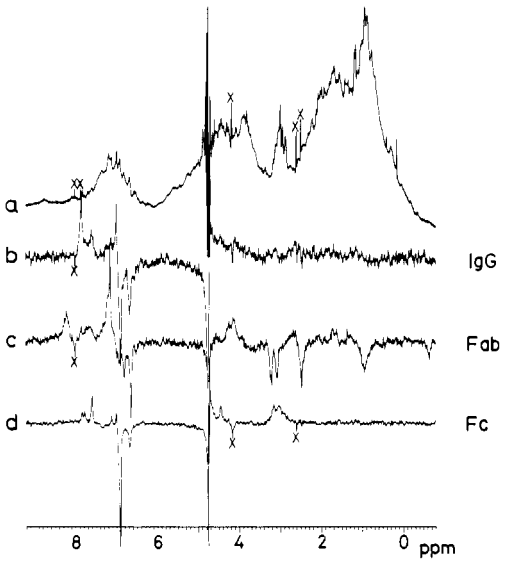

FIGURE 4: Photo-CIDNP difference spectra of intact IgG1 protein Ike-N and its Fab and Fc fragments: (a) normal spectrum of intact IgG1 Ike-N, 1000 scans; photo-CIDNP spectra of (b) intact IgG1 Ike-N, (c) Fab fragment, and (d) Fc fragment. The signals marked × indicate the polarizations due to flavin dye. Seven milligrams of intact IgG1 Ike-N and 5.3 mg of Fab and Fc fragments were dissolved in 0.3 mL of 0.2 M $NaCl/D_2O$. pH: (a and b) 7.50; (c) 7.20; (d) 7.20. In (b–d) 100 scans were accumulated with a laser irradiation time of 100 ms.

may have resulted in the significant decrease of the CIDNP polarization through *spin diffusion*. This point will be discussed later in relation to the CIDNP data obtained by using IgG proteins and their proteolytic fragments.

Immunoglobulin G. The photo-CIDNP spectrum of an IgG1 protein Ike-N is compared in Figure 4 with those of the Fab and Fc fragments that have been obtained from the intact protein by papain digestion (Endo & Arata, 1985); the spectrum of IgG1 Ike-N observed under the dark condition is also included in the figure. Most of the polarizations observed for the intact IgG1 protein and the Fab and Fc fragments correspond to the sharp signals that exist in the dark spectrum of the IgG1 protein. This suggests that the polarizations basically originate from the flexible parts of the molecules. In the following, assignments of some of the polarizations will be made on the basis of the results obtained by using a variety of Fab and IgG proteins.

Figure 4b shows that the intact IgG1 (pH 7.50) gives polarizations at 7.00 and 7.85 ppm. On the basis of the results of a series of our previous NMR studies, these signals can be assigned to the C2– and C4–H protons of His-224, which exists in the hinge region of the heavy chain (Arata et al., 1980; Endo & Arata, 1985; Ito & Arata, 1985). The hinge signals are observed at 7.15 and 8.23 ppm in the spectrum obtained by using the Fab fragment (pH 7.20) (see Figure 4c). This result again is consistent with our previous pH titration data obtained for the Fab fragment (Endo & Arata, 1985). We have previously shown that even in the case of the intact IgG protein *spin diffusion* does not affect the C2– and C4–H proton resonances of His-224, which is exposed to solvent and lacks in the vicinity an efficient source of the path for cross relaxation. In the case of the Fab fragment, we also observe two signals at 3.10 and 3.24 ppm originating from the two C_β protons of His-224. These aliphatic proton signals, which are directly polarized, are not detectable in the case of the intact IgG molecule. It is likely that in the case of IgG with a slower tumbling rate the growth of CIDNP signals is surpassed by an increased contribution from cross polarization. See also

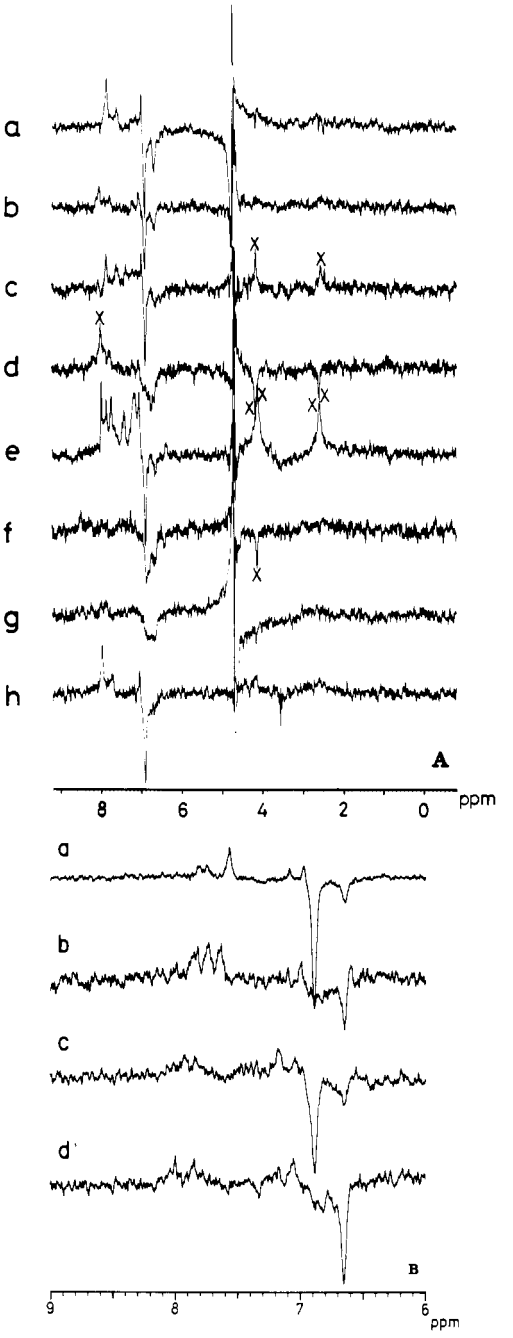

FIGURE 5: (A) Photo-CIDNP difference spectra of eight kinds of intact IgG proteins. IgG1: (a) protein Ike-N; (b) protein Yot; (c) protein Ogo. IgG2: (d) protein Ku-3. IgG3: (e) protein Her; (f) protein Jir. IgG4: (g) protein Kub. Human polyclonal IgG (h) was also used for the experiment. The signals marked × are polarizations due to the flavin dye. For (a) 7 mg, (b) 6.2 mg, (c) 5.0 mg, (d) 6.3 mg, (e) 5.6 mg, (g) 2.9 mg, and (h) 6.5 mg were dissolved in 0.3 mL of 0.2 M $NaCl/D_2O$. pH: (a) 7.50; (b) 7.18; (c) 7.29; (d) 7.21; (e) 7.16; (f) 5.71; (g) 7.31; (h) 7.13. One hundred accumulations were performed with a laser irradiation time of 50 ms for (c), (f), and (g) and of 100 ms for (a), (b), (d), and (e). (B) Photo-CIDNP difference spectra of Fc fragments of all subclasses: (a) Fc Ike-N (IgG1); (b) Fc In-K (IgG2); (c) Fc Jir (IgG3); (d) Fc RJ (IgG4). For (a) 5.2 mg, (b) 2.5 mg, (c) 1.8 mg, and (d) 1.9 mg were dissolved in 0.3 mL of 0.2 M $NaCl/D_2O$. pH: (a) 7.20; (b) 7.83; (c) 7.90; (d) 7.83. One hundred accumulations were performed with laser irradiation times of 100 ms for (a) and of 50 ms for (b–d).

the above discussion on the CIDNP results obtained for Bence Jones proteins.

Two Tyr polarizations are observed at 6.67 and 6.90 ppm in the spectrum of the intact IgG1. The Fc fragment also gives these two Tyr signals. Figure 5A compares photo-CIDNP

Table III: Amino Acid Substitutions Involving Tyr Residues in the Fc Region of All Subclasses of Human IgG and Mouse IgG2b Immunoglobulins[a]

	C_H2				C_H3				
	278	296	300	319	349	373	391	407	436
human									
IgG1	Tyr	Tyr	Tyr	Tyr	Tyr	Tyr	Tyr	Tyr	Tyr
IgG2	Tyr	Phe	Phe	Tyr	Tyr	Tyr	Tyr	Tyr	Tyr
IgG3	Tyr	Tyr	Phe	Tyr	Tyr	Tyr	Tyr	Tyr	Tyr
IgG4	Tyr	Phe	Tyr	Tyr	Tyr	Tyr	Tyr	Tyr	Tyr
mouse IgG2b	Phe	Tyr	Ile	Phe	Tyr	Asn	Tyr	Tyr	Tyr

[a] In the case of IgG3, a variety of allotypic substitutions are known. What is summarized in this table is valid for IgG3 proteins carrying G3m(g) and G3m(st) allotypes (van Loghem, 1978; van Loghem et al., 1982).

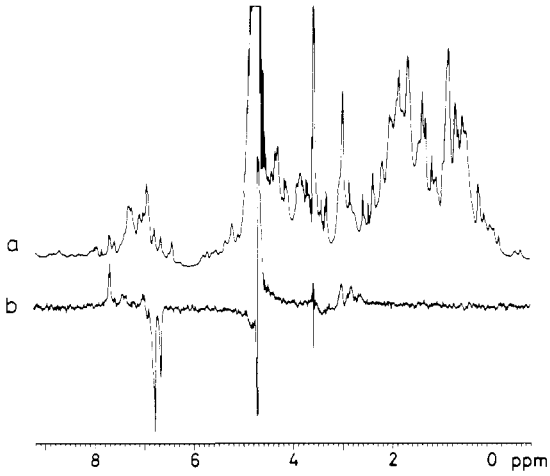

FIGURE 6: Normal (a) and photo-CIDNP difference (b) spectra of the pFc′ fragment obtained from IgG1 Ike-N. Two milligrams of the pFc′ fragment was dissolved in 0.3 mL of 0.2 M $NaCl/D_2O$. pH: 8.49. One thousand accumulations and 100 accumulations with a laser irradiation time of 50 ms were performed in obtaining the normal spectrum and the CIDNP spectrum, respectively.

spectra of seven kinds of human myeloma IgG proteins with subclasses ranging from IgG1 to IgG4. The spectrum obtained by using human polyclonal IgG is also included in the figure.

The presence or absence of the polarization observed at 6.90 ppm is quite characteristic of the subclasses of IgG. As Figure 5A shows, this signal is only observable in the case of IgG1 and IgG3 proteins. Protein Jir, which is a cryoglobulin, gives an unsatisfactory spectrum due to the low solubility. The limited availability of IgG4 protein Kub has resulted in the poor quality of its spectrum. The presence and absence of the 6.90 ppm signal in proteins with different subclasses can more clearly be seen in the spectra obtained by using Fc fragments (see Figure 5B). In Table III the amino acid substitutions involving Tyr residues in the Fc region are compared for all four subclasses of human IgG proteins. IgG3 proteins Her and Jir used in the present study are known to carry G3m(g) and G3m(st) allotypes, respectively (Michaelsen et al., 1977; Matsumoto et al., 1983). Inspection of Table III indicates that the Tyr signal observed at 6.90 ppm is due to C3- and C5-H protons of Tyr-296.

Another Tyr polarization at 6.67 ppm can be observed in the CIDNP spectra of IgG proteins of all human subclasses. Figure 6 reproduces a CIDNP spectrum of pFc′, which is the dimer of the C_H3 domain. The pFc′ gives three emission signals at 6.67, 6.82, and 6.97 ppm, which include the polarization observed at 6.67 ppm. This result indicates that the Fc signal observed at 6.67 ppm originates from the C_H3 domain. We have confirmed that this signal is missing in the case of mouse IgG2b (data not shown). Inspection of Table III indicates that the 6.67 ppm signal originates from Tyr-373.

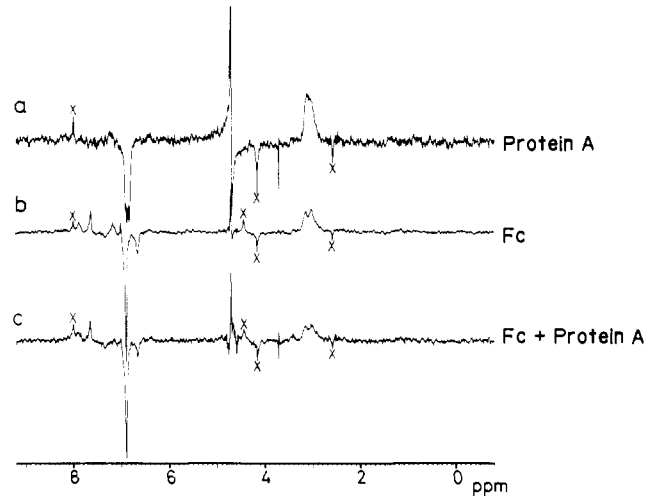

FIGURE 7: Photo-CIDNP difference spectra of protein A, Fc, and the protein A–Fc complex. Protein A used here is a mutant protein UV-2 that consists of D, A, and part of the B domains (Movitz et al., 1979). (a) One milligram of protein A was dissolved in 0.3 mL of 0.2 M $NaCl/D_2O$. One hundred accumulations were performed with a laser irradiation time of 50 ms. (b) Four milligrams of Fc obtained from IgG1 Ike-N and (c) 1 mg of protein A and 4 mg of Fc were dissolved in 0.3 mL of 0.2 M $NaCl/D_2O$. pH: (a) 7.18; (b) 7.17; (c) 7.18.

The C_H3 domain possesses five Tyr residues. According to the X-ray crystallographic structure of the Fc fragment (Deisenhofer, 1981), two of the five Tyr residues are shielded on association of the two C_H3 domains. Presumably three other Tyr residues, Tyr-373, Tyr-391, and Tyr-436, are responsible for the polarizations observed in the pFc′ fragment. A Tyr polarization that appears at 6.97 ppm in pFc′ apparently is not found in the Fc spectrum. The intensity of this signal is much less than that of the signals observed at 6.67 and 6.82 ppm. It is possible that this small signal is overlapped by the strong emission signal at 6.90 ppm. The signal observed at 6.82 ppm cannot be observed in the Fc spectrum. This suggests that the signal at 6.82 ppm originates from a Tyr residue that exists in the contact region of the C_H2 and C_H3 domains.

We also examined the effect of the binding of staphylococcal protein A to the Fc fragment that is obtained from IgG1 Ike-N. In the spectrum of protein A three Tyr polarizations are clearly observed, which completely disappear on complex formation (see Figure 7). The protein A used in the present experiment is a mutant protein UV-2 that contains domains D and A and part of domain B (Movitz et al., 1979). It is known that each domain of protein A contains one Tyr residues (Langone, 1982). The present photo-CIDNP results clearly indicate that each domain of protein A binds to the Fc region of the IgG and the Tyr residues are shielded on the complex formation. This is quite consistent with the result of the X-ray crystallographic study of the complex of Fc and F_B, a fragment corresponding to the B domain of protein A (Deisenhofer, 1981). As Figure 7 shows, the Fc signal observed at 6.67 ppm is still observable in the Fc–protein A complex, where a slight decrease in intensity but very little chemical shift change is observed. The crystal structure of the Fc–protein A complex shows that Tyr-373 is remote from the binding site. This result again is consistent with the above conclusion that the 6.67 ppm signal is due to Tyr-373.

Figure 4d shows that the Fc fragment obtained from IgG1 Ike-N gives three His polarizations. The Fc fragment of

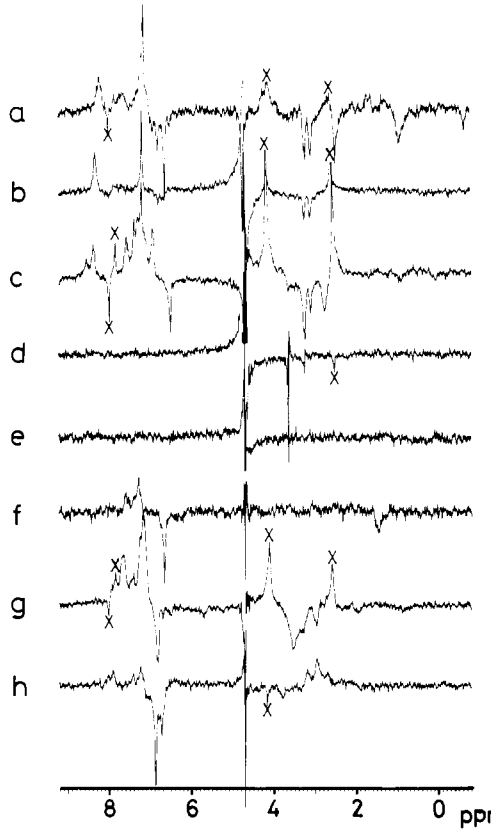

FIGURE 8: Photo-CIDNP difference spectra of eight kinds of Fab fragments. IgG1: (a) protein Ike-N; (b) protein Yot; (c) protein Ogo. IgG2: (d) protein Ku-3. IgG3: (e) protein Jir; (f) protein Gab; (g) protein Her. IgG4: (h) protein Kub. The polarizations marked × originate from the flavin dye. For (a) 5.5 mg, (b) 5.0 mg, (c, d) 4.2 mg, (e) 4.5 mg, (f) 3.1 mg, (g) 4.5 mg, and (h) 2.5 mg were dissolved in 0.3 mL of 0.2 M $NaCl/D_2O$. pH: (a) 7.20; (b) 7.17; (c) 7.13; (d) 7.23; (e) 7.18; (f) 7.33; (g) 7.20; (h) 7.18. One hundred accumulations were performed in all spectra. Laser irradiation times: 100 ms for (a–c, e); 50 ms for (d, f–h).

human IgG1 possesses six His residues, i.e., three at positions 268, 285, and 310 in the C_H2 region and three others at positions 429, 433, and 435 in the C_H3 region. NMR signals originating from the C2–H proton of all six His residues have been assigned (Shimizu et al., 1983; Matsuda et al., unpublished results). On the basis of the result of the assignments we are able to show that these signals originate from His-268, His-285, and His-433. Details of the assignments will be discussed in a later publication.

Eight kinds of Fab fragments were obtained from myeloma IgG proteins by limited papain digestion. The eight proteins comprise the heavy chains of all subclasses, i.e., IgG1 (proteins Ike-N, Yot, and Ogo), IgG2 (protein Ku-3), IgG3 (proteins Jir, Her, and Gab), and IgG4 (protein Kub). CIDNP spectra observed for these proteins are shown in Figure 8.

It was observed that the three IgG1 Fab give several distinctive polarizations. Except for the two large His C2– and C4–H proton polarizations, which originate from His-224 in the hinge region, other CIDNP signals are quite different from each other. The three Fab fragments used in the present measurements all possess the κ-type light chain, and therefore the difference in the amino acid sequences must originate from the hypervariable region.

In the case of Fab Ku-3 (IgG2) and Fab Jir (IgG3), no polarizations were observed, indicating that no exposed aromatic residues exist in both variable and constant regions of these proteins. This also indicates that the Fab fragments derived from other IgG3 proteins (proteins Her and Gab) show polarizations originating from the hypervariable region. Fab Kub (IgG4) gave several emission and absorption signals. Comparisons of the amino acid sequences of the C_H1 domain of IgG2 and IgG4 proteins indicate that there are only four substitutions, where no aromatic residues are included. This indicates that no polarizations are generated from the constant region of Fab Kub. In summary, the CIDNP data obtained for the Fab fragments indicate that except for His-224 of the $\gamma1$ chain all other aromatic residues observed occur in the hypervariable region.

Registry No. His, 71-00-1; Tyr, 60-18-4; Trp, 73-22-3.

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