Contribution of Tryptophan Residues to the Combining Site of a Monoclonal Anti Dinitrophenyl Spin-Label Antibody†

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ABSTRACT: Two Fab fragments of the monoclonal anti dinitrophenyl (DNP) spin-label antibody AN02 were prepared by recombination of specifically deuteriated heavy and light chains. In the recombinant H(I)L(II) all the tyrosines and phenylalanines were perdeuteriated as were the tryptophan residues of the heavy chain. In the recombinant H(II)L(I) all the tyrosines and phenylalanines were perdeuteriated as were the tryptophan residues of the light chain. Saturation of three resonances of H(I)L(II), assigned to tryptophan protons of the light chain, resulted in magnetization transfer to the aromatic proton at position 6 of the DNP ring and to the CH_2 protons of the glycines linked to the DNP in a diamagnetic hapten (DNP-DG). Saturation of three resonances of H(II)L(I) assigned to tryptophan protons of the heavy chain resulted in magnetization transfer to the CH_2 protons of the glycines in DNP-DG. From the dependence of the magnetization transfer on the irradiation time, the cross relaxation rates between the involved protons were estimated. The inferred distances between these protons of the hapten and certain tryptophan protons are 3–4 Å. It is concluded that in the combining site of AN02 there is one tryptophan from the light chain and one tryptophan from the heavy chain that are very near the hapten. When all tyrosines and phenylalanines were perdeuteriated and all tryptophan aromatic protons were deuteriated except for the protons at positions 2 and 5, titration of the Fab fragments with variable amounts of paramagnetic hapten showed that one proton from the light chain tryptophan is near (<7 Å) the unpaired electron and that three other protons are significantly closer than 15 Å. Eleven to fourteen tryptophan protons can be identified in the difference spectra, implying that there are five to seven tryptophans within 17 Å of the spin-label hapten. Amino acid sequences of the heavy and light chains were obtained by a combination of amino acid and DNA sequencing. A molecular model was constructed from the sequence data. A single binding site is apparent in the model. The two close tryptophans deduced from the magnetic resonance data are identified as tryptophan-91 of the light chain and tryptophan-47 of the heavy chain.

In previous studies we have shown that NMR spectroscopy combined with selective incorporation of deuteriated amino acids yields detailed information about the amino acid composition of the combining site of the monoclonal antibody AN02 (Anglister et al., 1984a; Frey et al., 1984). Recombination of specifically deuteriated heavy and light chains and calculation of the NMR difference spectra for the recombinant Fab fragments enabled us to assess the contributions of the heavy and light chain tyrosine residues to the combining site region (Anglister et al., 1985). Titration of the combining site with the spin-label hapten (SL) and calculation of difference spectra for the various occupancies of the combining site allowed us to determine distances of tyrosine residues from the unpaired electron (Anglister et al., 1984b). The applicability of this technique is limited by the magnitude of the off rate of the hapten. For AN02 we could determine distances in the range of 7–16 Å. Distances less than 7 Å could not be resolved. We have therefore undertaken magnetization transfer experiments to identify residues in the 2–4-Å range (Clore & Gronenborn, 1982, 1983). This technique should also identify residues that, due to the geometry of the hapten, are in contact with the hapten but are further than 7 Å from the unpaired spin. As in previous experiments, spectral overlap problems due to the over 2000 nonexchangeable protons were alleviated by incorporation of specifically deuteriated amino acids.

We have previously reported optical spectroscopic results which suggest that the dinitrophenyl (DNP) portion of the hapten is stacked on a tryptophan. Magnetization transfer experiments were therefore begun on samples deuteriated in phenylalanine and tyrosine. Magnetization transfer was detected and compared to results from spin-label titrations. Sequence data and computer modeling were used to suggest the location and geometry of the combining site.

The objective of our work is to use magnetic resonance methods and molecular modeling to develop a working model for interrelating combining site structure, binding constant, and on–off kinetics for AN02 as well as related monoclonal antibodies.

Materials and Methods

Synthesis of Dinitrophenyldiglycine (DNP-DG). 1,5-Difluoro-2,4-dinitrobenzene in ethanol was added to a 5-fold molar excess of glycine in 0.1 M $NaHCO_3$. The solution was

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stirred at room temperature in the dark overnight. After removal of the ethanol the product was precipitated by adding 1 M HCl to the solution at pH 2.0. The product was purified by recrystallization from water after DEAE chromatography in NH_4HCO_3 buffer. The product in diammonium salt form was confirmed by NMR and elemental analysis.

Synthesis of Trp-d_5. L-Tryptophan (500 mg) was suspended in 5 mL of D_2O, sonicated for 1 h, and then lyophilized. The dry tryptophan was added to 5 mL of D_2O and 300 μL of D_2SO_4, and the mixture was vacuumed and flushed with argon 3 times in a 40-mL vial with modified Mininert valves which connect to both an argon line and a vacuum pump. The vials were incubated in a 60 °C oven for 2 weeks in the dark and then chilled and neutralized with concentrated NH_4OH in an ice bath. The precipitate was collected by filtration and redissolved in 400 mL of MeOH, filtered, and dried by rotary evaporation. The dried material was redissolved in D_2O and lyophilized. The deuteriation procedure was repeated with this product. The final product was 98–99% deuteriated. The product was decolorized with activated charcoal. The yield was 60–75%. This method is a modification of the method of Griffiths et al. (1976).

Synthesis of 2,5-H-Trp. The synthesis of tryptophan protonated in the 2- and 5-positions and deuteriated in the 4-, 6-, and 7-positions (2,5-H-Trp) was done in 75% trifluoroacetic acid (TFA) as described by Matthews et al. (1977). (See Figure 8 for the numbering system used to identify hydrogen atoms on tryptophan.) Fifty-milligrams of Trp-d_5 was dissolved in 150 mL of H_2O and 300 mL of TFA (Sequenal grade). The mixture was vacuumed and flushed with argon twice and then incubated at room temperature in the dark for 40 h. The reaction mixture was diluted with H_2O and rotary evaporated until most of the solvent was removed. More H_2O was added, and the solution was neutralized with 1 N NH_4OH and rotary evaporated, redissolved in H_2O, and lyophilized. The dry product was decolorized as was Trp-d_5. At this stage the 2,5-H-Trp was not suitable for tissue culture; traces of volatile contaminants inhibited the growth of cells. The dry 2,5-H-Trp was resuspended, partially dissolved in ethanol (50 mg to 200 mL of ethanol), and rotary evaporated to dryness in the dark 4–5 times. The dry material was resuspended in a small volume of ethanol, and the precipitate was collected. The result of the NMR analysis of the product showed about 60% of the deuterium at the 2- and 5-positions was exchanged to hydrogen and less than 10% of the deuterium at other positions of the indole ring was exchanged.

Nuclear Magnetization Transfer (NMT) Experiments. Deuteriated anti-spin-label antibody (AN02) was derived by growth on specifically deuteriated amino acids, as described previously (Anglister et al., 1984a). Two preparations of Fab were obtained: Fab I was obtained from the hybridoma grown on perdeuteriated tryptophan, phenylalanine, and tyrosine; Fab II was obtained from the hybridoma grown on perdeuteriated phenylalanine and tyrosine. The chains were separated as previously described (Anglister et al., 1985). The heavy chain fragments and the light chains were recombined to produce the recombinants H(I)L(II) and H(II)L(I). The recombinants were dialyzed against 1 M propionic acid, twice against water, and then twice against 5 mM acetate buffer, pH 5.5. Three milliliters of 3 M tris(hydroxymethyl)aminomethane (Tris) buffer, pH 8.5, was added to each 10 mL of the Fab solution, and the obtained solution was then dialyzed against 10 mM borate buffer, pH 9. The solution was concentrated and dialyzed 4 times against borate buffer in deuterium oxide. The final concentration of H(I)L(II) was 3.4×10^{-4} M, and the concentration of DNP-DG was 7.8×10^{-3} M, which is 23 times the concentration of the Fab. The final concentration of H(II)L(I) was 3.6×10^{-4} M, and the concentration of DNP-DG was 5.4×10^{-3} M, which is 15 times the concentration of the Fab. A large excess of the hapten was necessary in order to get narrow lines for the protons of the free hapten. NMR spectra were taken with a JEOL 500-MHz spectrometer. Samples volumes were 550 μL. We collected 4000, 2500, 1700, and 1000 scans for irradiation times of 125, 250, 500, and 1000 ms, respectively. The NMT difference spectra were obtained in the following way. For a set of experiments with the same irradiation time but with different irradiation frequencies the free induction decays were collected in an interleaved manner, changing the irradiation frequency after 34 scans (the first 2 scans were not recorded). The free induction decays were Fourier transformed. The spectrum obtained when a proton resonance of the Fab was specifically irradiated was subtracted from a spectrum obtained when the irradiation frequency was outside the envelope of the resonances of the protein protons. Delays were 5.5 s, and the temperature was 45 °C.

DNA Sequencing. The light chain genomic clone was obtained from an *Mbo*I partial library in EMBL 4. A 2.8-kilobase fragment stretching from the *Hin*dIII site in the V_k – C_k intron to an upstream *Eco*RI site was subcloned into pUC 13. Sequencing was carried out with a synthetic primer specific for intron sequence just adjacent to J5. Sequencing reactions were carried out on base-denatured whole plasmid by using reverse transcriptase (Chen & Seeburg, 1985).

The heavy chain cDNA clone was obtained from a cDNA library made from AN02 poly(A) RNA. A primer specific for the heavy chain constant region, 5′-CACGACTGAGGCACC-3′, was used to prime the cDNA synthesis. These cDNAs were cloned into PUC 18 and positive clones selected by colony hybridization (Grunstein & Hogness, 1975). The positive inserts were sized on agarose gels and subcloned into M13 phage. These clones were then sequenced by the dideoxy method (Sanger et al., 1977; Biggin et al., 1983).

Amino Acid Sequencing. Automated Edman degradation was performed on Applied Biosystems gas-phase sequenators (Hewick et al., 1981). The results for the V regions of AN02 are summarized in Figure 1. Analysis of the phenylthiohydantoin-amino acids (PTH-amino acids) formed by conversion in methanolic HCl (Tarr, 1975) was essentially as described by Hunkapiller and Hood (1983). Degradation of intact AN02 yielded only one sequence, that of the heavy chain, indicating the light chain was blocked.

Further analysis of both chains was achieved by the following methods. The Fab fragment of AN02 was reduced, carboxyamidomethylated, and separated into light (L) chain and Fd fragments. Digestion of the blocked L chain with calf liver pyroglutamate aminopeptidase (Sigma) followed by Edman degradation gave the sequence shown in Figure 1, demonstrating that the amino terminus was pyrrolidonecarboxylic acid. To restrict tryptic digestion to arginine, the substrates were succinylated (Glazer et al., 1975) or citraconylated (Atassi & Habeeb, 1972). The succinylated PTH-Lys cochromatographed with the *O*-methyl ester of PTH-Glu in the analytical system used. Although the trypsin (Worthington) was L-1-(tosylamido)-2-phenylethyl chloromethyl ketone (TPCK) treated, some peptides isolated were the result of chymotryptic cleavage (Titani et al., 1982). In the case of the L chain, peptides were aligned by homology with other murine V_k sequences (Kabat et al., 1983). For the Fd fragment, overlapping peptides from CNBr cleavage (Spande et

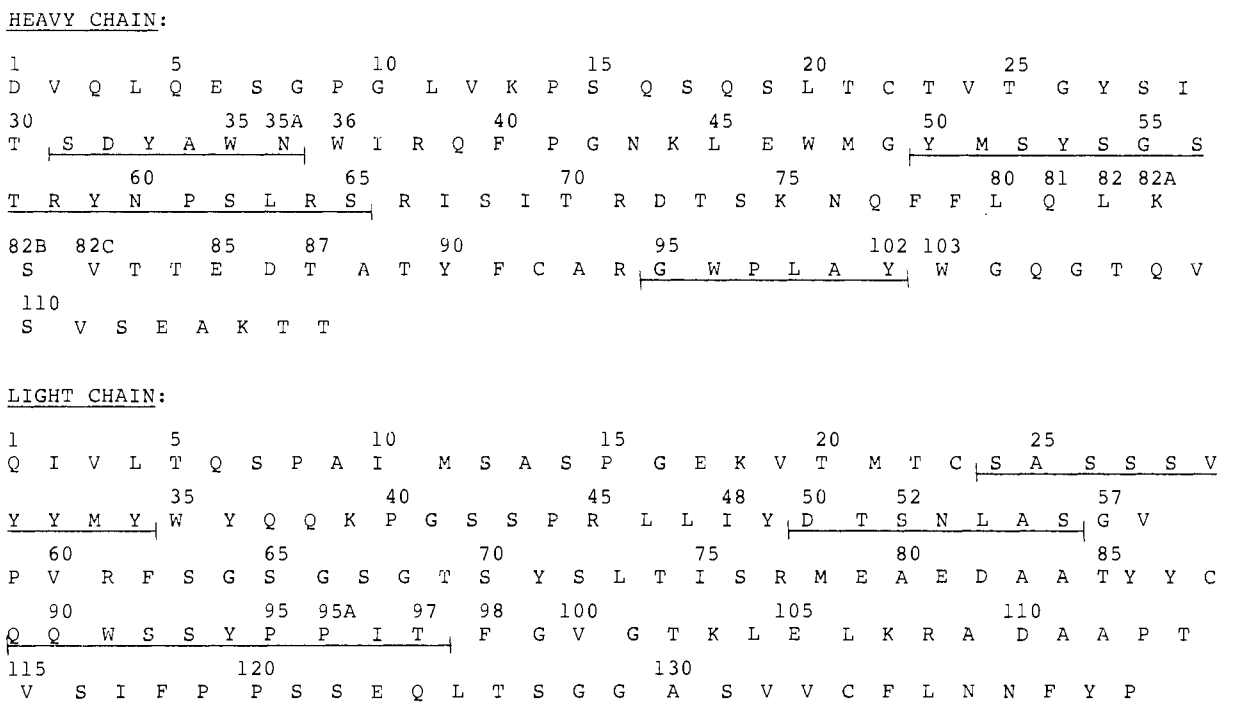

FIGURE 1: Amino acid sequence N-terminal residues of the heavy and light chains of monoclonal antibody AN02. The sequence was determined by both amino acid and DNA sequencing methods. Hypervariable regions are underlined.

al., 1970) or staphylococcal protease (Worthington) digestion (Drapeau et al., 1972) were isolated. Reverse-phase high-pressure liquid chromatography (RP-HPLC) was performed on C4 or C18 columns (10-μm particle size, 0.46 × 25 cm) from Vydac (Separations Group) under the conditions given in Table I.

Titration with Paramagnetic Hapten. The paramagnetic hapten titration of the AN02 antibody labeled with per-deuteriated tyrosine, perdeuteriated phenylalanine, and 2,5-H-Trp was carried out by using methods similar to those described elsewhere (Anglister et al., 1984b).

Molecular Modeling. The molecular model for the variable chain dimer of the AN02 antibody is based on the known X-ray structures of four Fab fragments: MPC603 (Segal et al., 1974), NEW (Saul et al., 1978), KOL (Marquart et al., 1980), and J539 (Won Suh et al., 1986). The first step of the completely automated modeling involves alignment of all five variable dimer sequences. This is done by dividing each sequence into 28 structural segments chosen to correspond to the framework strands, the loops, and the hypervariable loops. It is easy to decide which residues are in which segment as the framework segments are very homologous and the residues in the loops are simply those not in the framework. For every segment of AN02 there are four matching segments, one from each of the four known variable chain dimers. Choice of the single "best" match is made as follows: If any of the four segments is exactly the same length as the corresponding AN02 segment, then select the segment with the highest sequence homology to the AN02 segment. If there are no segments of the same length, then introduce a single gap (an insertion or a deletion) at a position chosen both to maximize homology and to have a short chain break at the gap. This alignment scheme, which will be described and evaluated elsewhere (Levitt, unpublished results), is different from that used to model the anti-lysozyme antibody D1.3 (Chothia et al., 1986). The present method does better on D1.3, reducing the main chain root mean square deviation between the model and X-ray structure for the hypervariable loop L1 from 3.76 to 1.09 Å.

The final alignment for AN02 consists of 17 segments of residues. The light chain consists of residues 1–9 from J539, 10–15 from MPC603, 16–33 from J539, 34–39 from MPC603, 40–46 from NEW, 47–88 from J539, 89–98 from KOL, and 99–107 from J539. The heavy chain consists of residues 1–26 from NEW, 27–33 from MPC603, 34–44 from KOL, 45–61 from NEW, 62–77 from J539, 78–92 from NEW, 93–97 from KOL, 98–108 from J539, and 105–112 from MPC603. There is a one-residue insertion at position 33 of the heavy chain, but all other AN02 residues are matched to a residue of one of the known structures.

Before the known coordinates of these four antibodies are used to model AN02, each set of coordinates is rotated into a common coordinate system (here arbitrarily chosen to be that of the KOL antibody). Coordinates for AN02 are taken as those of the equivalent atoms in the match residue; no attempt is made to preserve the continuity of the chain. When a residue of AN02 is larger than the equivalent residue, coordinates will not be available for all the atoms. Here, these missing atoms are placed at random within a sphere of radius 1 Å from the known atom to which they are bonded. The AN02 model has 89 missing atoms out of a total of 1781 atoms (not including all the hydrogen atoms). Energy minimization (Levitt, 1983) is used to improve the stereochemistry of the model by eliminating close contacts, forming strong hydrogen bonds, and ensuring that bond lengths, bond angles, and torsion angles are close to their standard, accepted values (after 600 steps of conjugate gradient minimization, the total energy drops from 1×10^{10} to −1589 kcal/mol).

RESULTS

Figure 1 shows the amino acid sequences found in this work. The light chain V gene is very similar to that of the anti-phenyloxazolone hybridoma NQ2-6.1 (Berek et al., 1985). The J segment used is J5. The heavy chain V gene is similar to MOPC 315 and to the anti-ARS (ARS = 4,4′-azodibenzenearsonic acid) hybridoma 36-60 (Kabat et al., 1983). The J segment used is probably J3 but contains three point mutations. The hypervariable regions are underlined.

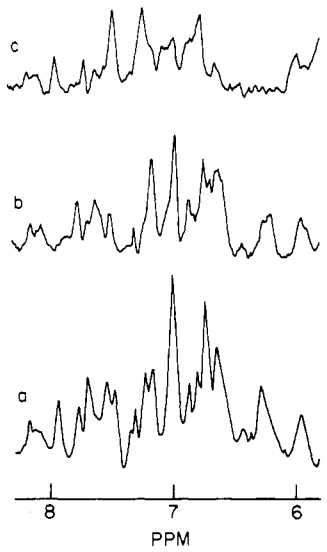

FIGURE 2: NMR difference spectra Fab(DNP-DG) – Fab(SL) giving tryptophan proton resonance signals in the combining site region: (a) Tryptophan protons from both chains contribute to the difference spectrum. (b) Reconstituted recombinant H(II)L(I). The difference spectrum shows the contributions of tryptophan protons from the heavy chain. (c) Reconstituted recombinant H(I)L(II). The difference spectrum shows the contributions of tryptophan protons from the light chain.

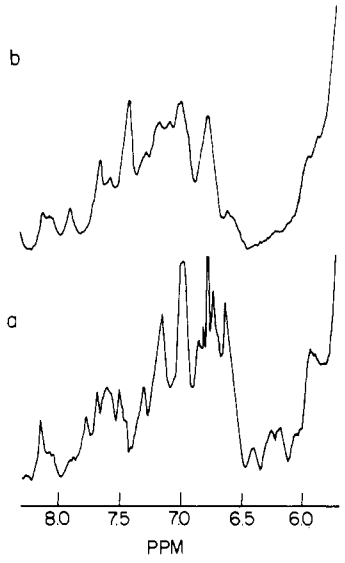

FIGURE 3: Proton NMR spectrum of two different reconstituted recombinant Fab fragments. (a) NMR spectrum of H(II)L(I) in which tyrosine and phenylalanine as well as the tryptophan residues of the light chain are perdeuteriated. (b) NMR spectrum of H(I)L(II) in which tyrosine and phenylalanine as well as the tryptophan residues of the heavy chain are perdeuteriated.

Figure 2 shows difference spectra Fab(DNP-DG) – Fab(SL) for the antibody labeled with perdeuteriated phenylalanine and tyrosine and for the recombinants H(I)L(II) and H(II)L(I) (see Materials and Methods). It can be seen that the tryptophan signals from the light chain and the tryptophan signals from the heavy chain sum to the difference spectrum for all of the tryptophans in the combining site region. Figure 3 shows the proton resonance spectra arising from all tryptophans in the heavy chain (Figure 3a) and all tryptophans in the light chain (Figure 3b). Figures 2b and 3a are very similar, since five of the six heavy chain tryptophans are in the V domain in the combining site region.

In order to simplify the tryptophan resonance spectra, we employed tryptophan that was protonated in the 2- and 5-positions and deuteriated in the 4-, 6-, and 7-positions. Illustrative titration spectra are given in Figure 4. In the Fab(0) – Fab(SL) spectrum there are 11–14 resonances, and thus there are 5–7 tryptophans in the combining site region. Our previous estimate of the number of tryptophans in the combining site region was low due to poor resolution of the signals (Anglister et al., 1984a). The very rapid appearance of the signal near 7.93 ppm shows that this proton is within 7 Å of the unpaired electron (Anglister et al., 1984b).

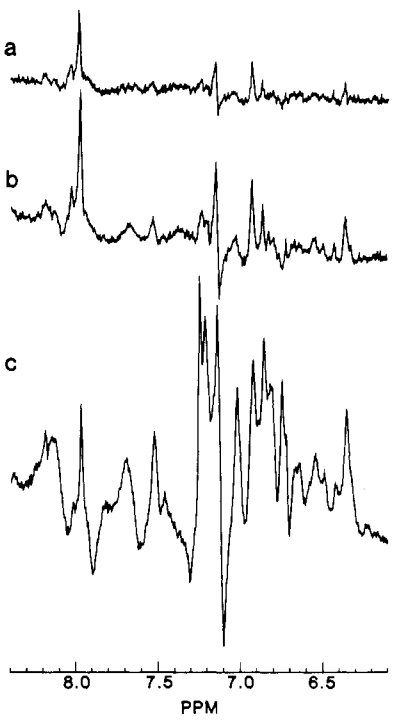

FIGURE 4: Illustrative titration difference spectra: Fab(0) – Fab (SL) and Fab(DNP-DG) – Fab(SL). Signals are due to protons in the 2- and 5-positions of tryptophan, the other positions being deuteriated. Fab(0) – Fab(SL) for (a) 6.8% and (b) 100% occupancy of the combining site. Fab(DNP-DG) – Fab(SL) for (c) 100% occupancy of the combining site.

Two types of nuclear magnetization transfer from protein to diamagnetic hapten (DNP-DG) were observed. When the sample was irradiated anywhere in the region 6.5–9.0 ppm, there was a decrease in the intensity of the sharp line at 9.15 assigned to proton 3 between the two nitro groups on the DNP ring. This is due to chemical transfer of nuclear magnetization from bound hapten to free hapten. This was verified by irradiating at various frequencies throughout the aromatic region with samples in which tryptophan phenylalanine and tyrosine were all perdeuteriated. For samples with this deuteriation there is no NMT to free hapten proton resonances at 5.5 or 3.9 ppm, but transfer to the signal at 9.15 ppm is always observed. The chemical exchange of DNP-DG between bound and free states evidently results in a lifetime-broadened resonance of aromatic proton 3 which includes the range 6.5–9.0 ppm. This interpretation was tested by showing that the transfer signal at 9.15 depends on the irradiating power (see Figure 5). It was also noted that the line width of the free signal of proton 3 increases roughly in proportion to the reciprocal of the free hapten concentration, as expected for slow exchange.

Nuclear magnetization transfer due to dipole–dipole coupling was observed between tryptophan proton resonances and the methylene protons of DNP-DG and between tryptophan protons and the aromatic 6-proton of DNP-DG. Illustrative NMT spectra are given in Figure 6. Some intramolecular NMT within tryptophan residues was observed, as illustrated in Figure 6a.

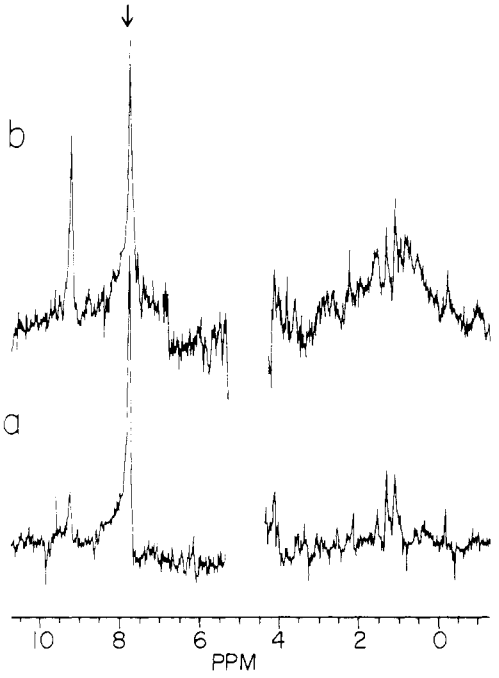

FIGURE 5: NMT from the aromatic proton at position 3 of DNP-DG in the bound state to the free state, due to chemical exchange. Tryptophan and phenylalanine are perdeuteriated, and tyrosine is deuterated in the 2- and 6-positions. The irradiation power employed in spectrum b is 6.3 times higher than the irradiation power employed in spectrum a. The point of irradiation is indicated by the arrow, but similar results are obtained for irradiation throughout the region of 6.5–9.0 ppm.

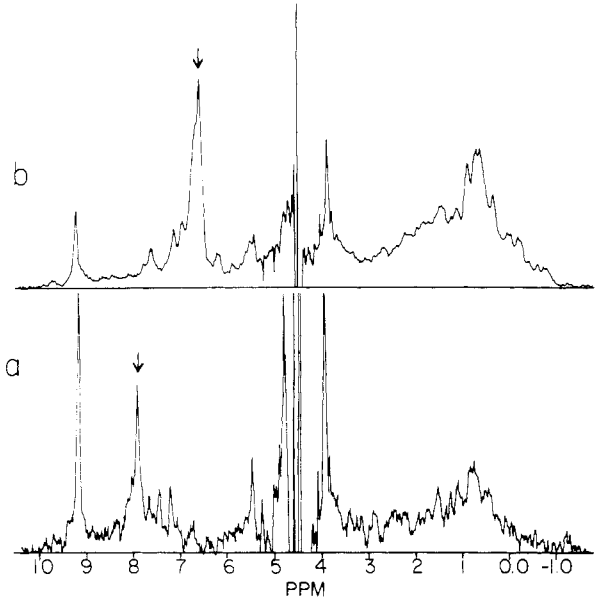

FIGURE 6: NMT difference spectra resulting from saturation of resonances of the recombinant H(I)L(II). (a) The dipole–dipole NMT is from a light chain proton of tryptophan (Trp-91) at 7.93 ppm to the CH_2 groups of DNP-DG at 3.95 ppm and to the aromatic proton at position 6 of DNP at 5.5 ppm. The NMT to the aromatic proton at position 3 of DNP at 9.15 ppm is due to chemical exchange between bound and free hapten. The signals at 7.46 and 7.2 ppm are probably due to intramolecular dipole–dipole NMT within Trp-91. (b) The dipole–dipole NMT is from a tryptophan residue of the heavy chain (probably Trp-47) to the CH_2 groups of DNP-DG. The NMT to the aromatic proton at 9.15 ppm is due to chemical exchange.

In order to ensure that the NMT observed was direct rather than indirect (spin diffusion), the nuclear magnetization transfer was measured by using various irradiation periods as illustrated in Figure 7. All the specific transfers reported in the present work were linear at short times. Table I gives a

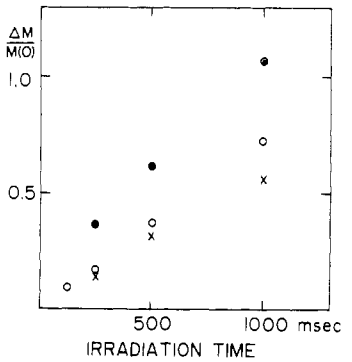

FIGURE 7: Buildup of NMT with increasing irradiation time due to dipole–dipole coupling. The symbols ○, ●, and × refer to irradiation of Trp protons at 7.93, 7.2, and 7.46 ppm, respectively. Transfer is to the DNP proton resonance at 5.5 ppm (aromatic proton 6). Here, $\Delta M/M(0)$ is the change in the aromatic proton signal at 5.5 ppm in units of the magnetization of a single proton.

Table I: Estimated Distances from Nuclear Magnetization Transfer

irradiated tryptophan (ppm)	chain	nucleus receiving magnetization transfer	estimated distances[a] between nuclei (Å)
7.2	light	H6(DNP)	3.4 ± 0.7
7.2	light	CH_2(Gly)	3.5 ± 0.7
7.5	light	H6(DNP)	3.5 ± 0.7
7.5	light	CH_2(Gly)	3.6 ± 0.7
7.93	light	H6(DNP)	3.0 ± 0.7
7.93	light	CH_2(Gly)	3.2 ± 0.7
7.2	heavy	H6(DNP)	3.9 ± 0.8
7.2	heavy	CH_2(Gly)	3.9 ± 0.8
7.0	heavy	H6(DNP)	3.9 ± 0.8
7.0	heavy	CH_2(Gly)	3.95 ± 0.7
6.6	heavy	CH_2(Gly)	3.7 ± 0.7

[a] The calculation of error was made by calculating the distance for two extreme cases. (1) Assume that with a given radiation frequency four proton are irradiated while only one of them is responsible for the magnetization transfer. In this case, the actual relative cross relaxation is 4 times larger, and therefore the calculated distance is 20% shorter. (2) From the difference spectra we conclude that the line width of the tryptophan proton is usually roughly equal to the line width of the hapten protons. If the line width of the tryptophan protons is as large as 3 times the line width of the protons of the hapten, the relative cross relaxation may then be 3 times smaller and the distances 20% larger.

list of these nuclear magnetization transfers together with estimated distances. The estimated distances are based on the following considerations.

The initial rate of change in the magnetization of the free hapten M_H is given by

$$dM_H/dt = -\sigma M_F(0) \quad (1)$$

where $M_F(0)$ is the equilibrium magnetization of a single Fab proton. For a large ratio between the concentration of the free hapten and the concentration of the bound hapten, and when the exchange is fast relative to T_1, the total magnetization of the hapten is approximately equal to the magnetization of the free hapten. An accurate evaluation of the cross relaxation parameter σ is difficult because of problems of normalization. In the NMT difference spectra the irradiated proton signal may arise from more than one proton. From the difference spectra we observe that the line widths of the protons of the tryptophans are roughly the same as the line widths of the free hapten protons when the hapten is in large excess. For simplicity we assume therefore that in each experiment only one proton is irradiated and that the line width of the free DNP-DG proton is the same as the line width of the tryptophan proton. In such cases the relative change in the peak height is the relative change in the magnetization, and we can use

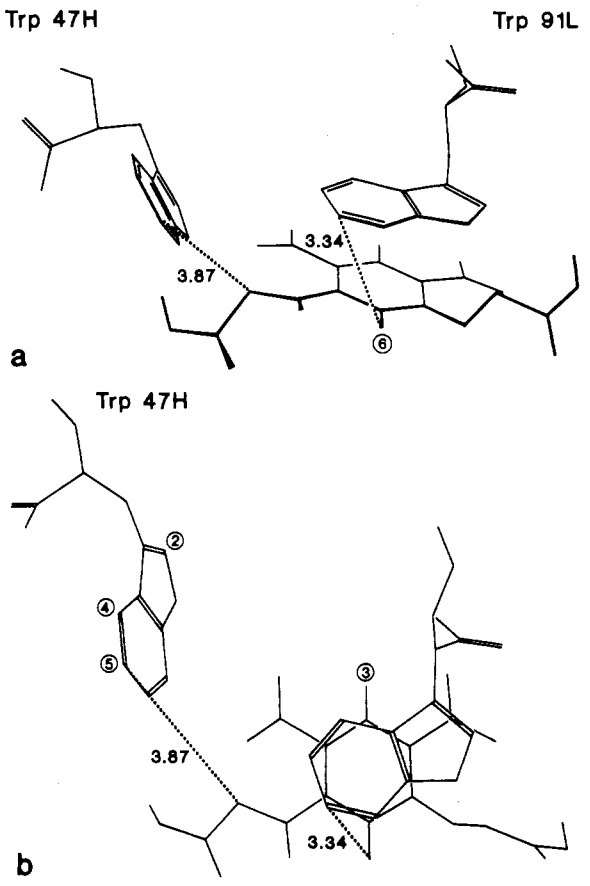

FIGURE 8: Computer graphics model showing the diamagnetic hapten in relation to Trp-91 of the light chains and Trp-47 of the heavy chain of AN02. Interatomic distances are indicated by dotted lines. Numbers by dotted lines give distances in angstroms between protons in the diamagnetic hapten and carbon atoms of tryptophan residues. Numbers in circles give numbering system used for carbon and hydrogen atoms for tryptophan and for the aromatic ring of the diamagnetic hapten.

eq 1 using peak heights in the difference spectra. From the initial slopes of the curves shown in Figure 7 we can calculate the cross relaxation rates and evaluate the distances between the protons. The rotational correlation time of the Fab is reported to be 2×10^{-8} s (Anglister et al., 1984a).

Discussion

The NMR data in the present work demonstrate that in the AN02 antibody there are five to seven tryptophan residues in the combining site region; that is, there are five to seven tryptophan residues within 17 Å of the unpaired spin in the paramagnetic hapten. The nuclear magnetization transfer data obtained with a diamagnetic hapten show that two of these tryptophan residues are close (within 3–5 Å) to the protons of the diamagnetic hapten. Model building indicates that the closest tryptophan residue seen in the NMR must be tryptophan-91 on the light chain. Our model has a region with a diameter of the order of 10–20 Å within which many of the aromatic residues are localized. Within this region a pocket or indentation containing tryptophan-91 from the light chain is the most obvious candidate for a combining site. The greatest uncertainty is the identification and location of the second tryptophan residue found by NMR (the tryptophan residue of the heavy chain). This second residue is probably tryptophan-47 from the heavy chain on the basis of our model building.

An illustrative configuration of tryptophan-91 of the light chain (Trp-91L), tryptophan-47 of the heavy chain (Trp-47H), and the diamagnetic hapten is shown in Figure 8. The model was constructed without any reference to the hapten. From the magnetic resonance data we conclude that the proton at aromatic position 6 of DNP-DG is within 3–4 Å of proton 5 of Trp-91L. This conclusion is based on the following facts: (i) There is dipole–dipole NMT from the light chain proton signal at 7.93 ppm to aromatic proton 6 of DNP-DG. (ii) There is no heavy chain proton signal at 7.93 ppm. (iii) The 2,5-H-Trp spectrum has a signal at 7.93 ppm. (iv) This proton is closest to the paramagnetic hapten on the basis of the titration. (v) Since the signal at 7.93 ppm is present in the 2,5-H-Trp spectra, it must be a proton in position 2 or 5 of tryptophan on the light chain, and Trp-91L is the unique choice on the basis of the model. (vi) In Figure 6a one observes NMR between the signal at 7.93 ppm and other Trp protons. The simplest interpretation is that this is intramolecular NMT, in which case the proton must be proton 5 of Trp-91L, since proton 2 is not close to any other proton in this residue. The position of the DNP ring in Figure 8 was chosen to satisfy this proximity of proton 5 of Trp-91L and aromatic proton 6 of DNP-DG. The CH_2 protons of DNP-DG are not shown in Figure 8. With DNP-DG in the combining site, one pair of CH_2 protons is within 3–5 Å of protons on Trp-91L and the other pair of CH_2 is within 3–5 Å of protons on Trp-47H. Thus the molecular geometry in Figure 8 is consistent with the observed NMT from tryptophans to DNP-DG.

We previously observed changes in the absorption spectrum of DNP-glycine upon binding to AN02. Such changes in the absorption spectrum of DNP are well-known and are due to stacking interactions between the DNP ring and tryptophan (Little & Eisen, 1967). In separate work we have recently obtained refined distances between the unpaired electron and 10 tyrosine residues (to be published). These data plus additional NMT between other amino acids and the diamagnetic hapten should provide sufficient constraints on the model building to provide a useful picture of the combining site structure.

References

Anglister, J., Frey, T., & McConnell, H. M. (1984a) *Biochemistry 23*, 1138–1142.

Anglister, J., Frey, T., & McConnell, H. M. (1984b) *Biochemistry 23*, 5372–5375.

Anglister, J., Frey, T., & McConnell, H. M. (1985) *Nature (London) 315*, 65–67.

Atassi, M. Z., & Habeeb, A. F. S. A. (1972) *Methods Enzymol. 25*, 546–553.

Berek, C., Griffiths, G. M., & Milstein, C. (1985) *Nature (London) 316*, 412–418.

Biggin, M. D., Gibson, T. J., & Hong, G. F. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 3963–3965.

Chen, E., & Seeburg, P. H. (1985) *DNA 4*, 165–170.

Chothia, C., Lesk, A., Levitt, M., Amit, A. G., Mariuzza, R. A., Phillips, S. E. V., & Poljak, R. J. (1986) *Science (Washington, D.C.) 233*, 755–758.

Clore, G. M., & Gronenborn, A. M. (1982) *J. Magn. Reson. 48*, 402–417.

Clore, G. M., & Gronenborn, A. M. (1983) *J. Magn. Reson. 53*, 423–442.

Drapeau, G. R., Broily, Y., & Houmaed, J. (1972) *J. Biol. Chem. 247*, 6720–6726.

Frey, T., Anglister, J., & McConnell, H. M. (1984) *Biochemistry 23*, 6470–6473.

Glazer, A. N., Delange, R. J., & Sigman, D. S. (1975) in *Chemical Modification of Proteins*, p 78.

Griffiths, D. V., Feeney, J., Roberts, G. C. K., & Burgen, A. S. V. (1976) *Biochim. Biophys. Acta 446*, 479–485.

Grunstein, M., & Hogness, D. S. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3961-3965.
Hewick, R. M., Hunkapillar, M. W., Hood, L. E., & Dreyer, W. J. (1981) *J. Biol. Chem. 256*, 7990-7997.
Hunkapillar, M. W., & Hood, L. E. (1983) *Methods Enzymol. 91*, 486-493.
Kabat, E. A., Wu, T. T., Bilofsky, H., Redi-Miller, M., & Perry, H. (1983) *Sequences of Proteins of Immunological Interest*, National Institutes of Health, Bethesda, MD.
Levitt, M. (1983) *J. Mol. Biol. 170*, 723-764.
Little, J. R., & Eisen, H. N. (1967) *Biochemistry 6*, 3119-3125.
Marquart, N., Diesenhofer, J., Huber, R., & Palm, W. (1980) *J. Mol. Biol. 141*, 369-391.
Matthews, J. R., Matthews, K. S., & Opella, S. J. (1977) *Biochim. Biophys. Acta 497*, 1-13.
Sanger, F., Nicklen, S., & Coulsen, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463-5468.
Saul, R., Amzel, L., & Poljak, R. (1978) *J. Biol. Chem. 253*, 585-597.
Segal, D., Padlan, E., Cohen, G. H., Rudikoff, S., Potter, M., & Davies, D. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 4298-4302.
Spande, T. F., Witkop, B., Degan, Y., & Patchornik, A. (1970) *Adv. Protein Chem. 24*, 97-260.
Tarr, G. E. (1975) *Anal. Biochem. 63*, 361-370.
Titani, K., Sasagawa, T., Resing, K., & Walsh, K. A. (1982) *Anal. Biochem. 123*, 408-412.
Won Suh, S., Bhat, T. N., Navia, M. A., Cohen, G. H., Rao, D. N., Rudikoff, S., & Davies, D. (1986) *Proteins: Struct., Funct., Genet. 1*, 74-80.

Antibodies Directed against N-Terminal Residues on Actin Do Not Block Acto-Myosin Binding†

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ABSTRACT: Several studies using a variety of approaches have suggested a possible role for the amino-terminal residues of skeletal muscle actin in acto–myosin interaction. In order to assess the significance of acto–S-1 contacts involving the N-terminal segment of actin, we have prepared polyclonal antisera against a synthetic peptide corresponding to the seven amino-terminal residues of rabbit skeletal muscle actin (α-N-terminal peptide). Affinity-purified immunoglobulin (Ig) G (and Fab) prepared from these antisera reacts strongly and specifically with the amino-terminal segment of both G- and F-actin but not with myosin subfragment 1 (S-1). This specificity was determined by Western blot analysis of actin and its proteolytic fragments and the inhibition of the above reactivity by the α-N-terminal peptide. The α-N-terminal peptide did not interact with S-1 in solution, affect S-1 and actin-activated S-1 MgATPase, or cause dissociation of the acto–S-1 complex. In separate experiments F-actin could be cosedimented with S-1 and affinity-purified IgG or Fab by using an air-driven ultracentrifuge. Densitometric analysis of sodium dodecyl sulfate/polyacrylamide gels of pellet and supernatant fractions from such experiments demonstrated the binding of both S-1 and IgG or Fab to the same F-actin protomer. Our results suggest that, while the acidic N-terminal amino acids of actin may contact the myosin head, these residues cannot be the main determinants of acto–S-1 interaction.

A common goal in electron microscopy and solution studies of acto–subfragment 1 (acto–S-1) is to elucidate, albeit at different resolutions, the structure of the actomyosin interface and the changes in this interface during cyclical interaction of myosin cross-bridges with actin. Low-resolution electron microscopy and image reconstruction work resulted in a three-dimensional model of the acto–S-1 complex (Moore et al., 1970; Wakabayashi & Toyoshima, 1981; Taylor & Amos, 1981; Amos et al., 1982) but could not determine the regions of protein–protein contact between actin and S-1. This goal is now being vigorously pursued in solution studies.

One approach to the investigation of protein–protein contacts along the acto–S-1 interface in solution has been the chemical cross-linking of actin to S-1. Mornet et al. (1981) cross-linked actin to the 50- and 20-kDa tryptic fragments of S-1 using the zero-length cross-linker 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC). The elegant studies of Sutoh (1982a,b, 1983) showed further that the acidic N-terminal residues of actin (Vandekerckhove & Weber, 1978a–c; Lu & Elzinga, 1977; Elzinga & Lu, 1977) can be cross-linked to either the 20- or 50-kDa fragments and that C-terminal residues of actin interact with LC-1. Consequently, it has been assumed that the N-terminal residues of actin are an important component of the myosin binding site on actin and contribute to the electrostatic attraction between these proteins. However, the assumption that the carbodiimide cross-linking sites can be equated with acto–S-1 binding sites

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