Affinity-Labeled Peptides Obtained from the Combining Region of Myeloma Protein 460. I. Heavy-Chain-Labeling Patterns Using Dinitrophenyl Azide Photoaffinity Label†

John Lifter, Choy-Leong Hew, Masanori Yoshioka, Frank F. Richards,* and William H. Konigsberg

ABSTRACT: A mouse IgA myeloma protein, protein 460, which binds the 2,4-dinitrophenyl (Dnp) group was reacted with two photoaffinity labeling reagents, Dnp-alanyl diazoketone (which we have previously shown to label residue 54 in the light chain) and Dnp-N_3. The residues that react with Dnp-N_3 are located in two separate fragments derived from the heavy-chain-variable region and were obtained after cleaving partially reduced and alkylated heavy chain with cyanogen bromide. The modified amino acid residues were found both in the amino terminal cyanogen bromide fragment (residues 1–34) and in another variable region fragment (residues 84–109). These fragments were linked by a disulfide bridge. After complete reduction and alkylation, the fragments spanning these two regions were separated by ion exchange chromatography. A tyrosine residue was labeled in each fragment and these residues were assigned to position 33, by automatic sequencing, and to position 88 by comparison with the known sequence of MOPC 173 heavy chain.

2,4-Dinitrophenylalanyl diazoketone (Dnp-AD)[1] (Converse and Richards, 1969) and dinitrophenyl azide (Dnp-N_3) (Yoshioka *et al.*, 1973) are photoactivated affinity labeling reagents which share the same haptenic determinant, the dinitrophenyl ring, but have different reactive groups. We have used both of these reagents to label the combining region of protein 460. Dnp-AD reacted almost exclusively with a lysine residue located at position 54 of the protein 460 light chain (Hew *et al.*, 1973). In contrast, 85% of the Dnp-N_3, attached by covalent bonds to protein 460, was found in the Fd region (Yoshioka *et al.*, 1973). The remainder, 15%, of the covalently attached reagent was found in the light chain and was sufficient to permit its partial localization (Hew *et al.*, 1973). This paper describes the results which allowed us to assign the positions occupied by the Dnp-N_3 labeled residues in the heavy chain.

Materials and Methods

Purification and Labeling of Protein 460. The purification of protein 460 and the synthesis of [^{3}H]Dnp-N_3 have been described previously (Yoshioka *et al.*, 1973). Protein 460 (3.35×10^{-5} M) was irradiated in the presence of 2.9×10^{-4} M [^{3}H]Dnp-N_3 as described in Yoshioka *et al.* (1973). Approximately 1.3–1.5 mol of reagent was incorporated/mol of protein 460 for the preparation used in this study. A second irradiation in the presence of nonradioactive affinity labeling reagent, used previously to completely modify amino acid residues reactive with Dnp-AD (Hew *et al.*, 1973), was not employed.

Partial Reduction and Alkylation of Protein 460 and Isolation of Heavy and Light Chains. Partial reduction and alkylation were carried out by treating protein 460, 5 mg/ml, in 0.2 M Tris-HCl–0.075 M NaCl (pH 8.0) buffer with 0.01 M dithiothreitol and incubating 2 hr at room temperature. Subsequently, sodium iodoacetate was added to give a final concentration of 0.025 M. Heavy and light chains were isolated by gel filtration of partially reduced and alkylated protein 460 on Sephadex G-100 in 6 M urea–1 M propionic acid–0.1 M glycine. The isolated chains were dialyzed against 0.2 N acetic acid and lypholized.

CNBr Cleavage and Separation of CNBr Fragments. Partially reduced and alkylated heavy chains, 1 μmol, were reacted with 0.2–0.3 g of solid cyanogen bromide (Eastman Organic Chemicals, 919) in 5 ml of 70% formic acid for 24 hr at room temperature (Gross, 1967). The reaction mixture was diluted tenfold with water and lypholized. The products of the CNBr cleavage were separated by gel filtration on Sephadex G-75 in 6 M urea–1 M propionic acid–0.1 M glycine. The labeled fragment was completely reduced and alkylated in 6 M guanidine-HCl–0.5 M Tris-HCl–0.002 M EDTA (pH 9.0) buffer at 50° for 4 hr (Konigsberg, 1972). A 50-fold molar excess of dithiothreitol and a 100-fold molar excess of sodium iodoacetate were used for complete reduction and alkylation. The completely reduced and alkylated CNBr peptides were separated by gel filtration on Sephadex G-75 (6 M urea–1 M propionic acid–0.1 M glycine) followed by anion exchange chromatography on diethylaminoethyl-cellulose (DE52) using a Tris-HCl gradient from 0.01 to 0.2 M in 8 M urea (pH 8.0).

Sequencing Methods. The amino termini of peptides were identified by the dansyl procedure of Gray (1967; 1972) and the microdansyl-Edman method described by Weiner *et al.* (1972).

The automated sequencing of protein and peptides utilized a JEOL Model JAS-47K sequencer. A peptide program using dimethylallylamine buffer was employed; 300 nmol of heavy chain, or up to 1 μmol of peptide, was dissolved in 1 ml of anhydrous trifluoroacetic acid and transferred to the reaction cup. The subsequent operations were performed according to the JAS-47K Operating Manual (Japan Electron Optics Laboratory Co., Tokyo, Japan). Conversion of the thiazolinones to PTH-amino acids was accomplished using 1.0 N HCl for 10 min at 80°. The PTH-amino acids were extracted into ethyl ac-

† From the Departments of Molecular Biophysics and Biochemistry (J. L., C.-L. H., M. Y., and W. H. K.) and Internal Medicine and Microbiology (F. F. R.), Yale University School of Medicine, New Haven, Connecticut 06510. *Received February 15, 1974.* Supported by Grants GB-1655 from the National Science Foundation and GM 12607 and AI-08614 from the U. S. Public Health Service, and by the American Heart Association.

[1] Abbreviations used are: CNBr, cyanogen bromide; Dnp, 2,4-dinitrophenyl; Dnp-AD, 2,4-dinitrophenylalanyl diazoketone; Dnp-N_3, 2,4-dinitrophenyl azide; PTH, phenylthiohydantoin.

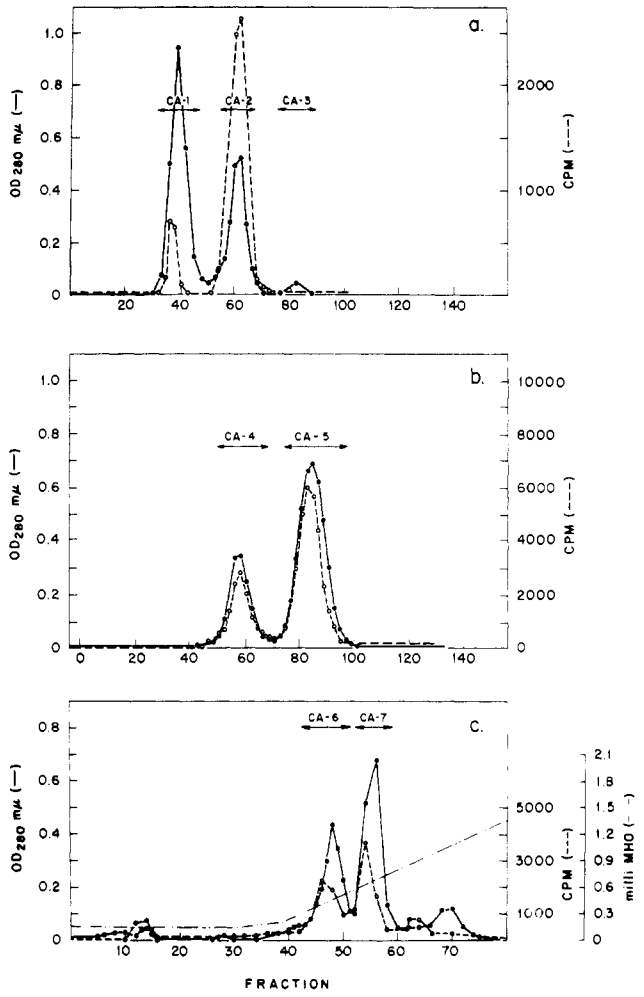

FIGURE 1: (a) Sephadex G-75 chromatography of CNBr cleaved partially reduced and alkylated Dnp-N_3 labeled protein 460 heavy chain. Solvent: 6 M urea–1 M propionic acid–0.1 M glycine. Column size: 2.5 × 150 cm. (b) Sephadex G-75 chromatography of completely reduced and alkylated CNBr fragment CA-2. The solvent and column size were the same as in Figure 1a. (c) DE52 anion exchange chromatography of CNBr fragment CA-5. Column size: 1 × 50 cm. Elution: two column volumes of starting buffer, 0.01 M Tris-HCl–8 M urea (pH 8.0), followed by a linear gradient from 0.01 M Tris-HCl–8 M urea, 400 ml (pH 8.0) to 0.2 M Tris-HCl–8 M urea, 400 ml (pH 8.0).

etate and identified either by gas–liquid chromatography on 10% DC560 columns in a Varian 1800 gas chromatograph (Pisano *et al.*, 1972), or by hydrolysis with 55% HI to free amino acids and identification by automated amino acid analysis (Smithies *et al.*, 1971; Inglis *et al.*, 1970).

Digestion of CNBr Fragment with Thermolysin. Digestion of CNBr peptides was performed with thermolysin (Matsubara *et al.*, 1965; Matsubara, 1966). The fragment was suspended in 0.2 M NH_4HCO_3 and thermolysin (Serva Feinbiochemica; diluted with sodium and calcium acetate) was added to a 1:50 (w/w) enzyme/fragment ratio. Digestion at 37° was allowed to proceed for 24 hr and was terminated by lyophilization.

Separation of Thermolytic Peptides. The thermolytic peptides were separated and purified by high-voltage electrophoresis at pH 1.9 and descending paper chromatography with 1-butanol–acetic acid–water–pyridine, 15:3:12:10.

Results

Preparation of Dnp-N_3 Labeled Heavy Chains from Protein 460. After protein 460 was reacted with [^{3}H]Dnp-N_3 (Yoshioka *et al.*, 1973), 0.55 mol of reagent was incorporated/mol of heavy chain, the partially reduced and alkylated heavy chains were treated with cyanogen bromide, and the fragments were separated as described below.

TABLE I: Amino Acid Composition of Dnp-N_3 Labeled Peptides.

Residue	CA-6	CA-7
Lysine	1	1
Arginine	3	0
CM-cysteine	1	1
Aspartic	4	2
Threonine	2	4
Serine	3	6
Glutamic	1	5
Proline	*a*	2
Glycine	3	3
Alanine	2	0
Valine	1	3
Isoleucine	4	1
Leucine	1	4
Tyrosine	3	1
Phenylalanine	3	0
Homoserine	1	1
	33	34

[a] Not determined.

Separation of Dnp-N_3 Labeled CNBr Fragments. The CNBr fragments were fractionated on a Sephadex G-75 column, in 6 M urea, giving the elution profile shown in Figure 1a. Most of the radioactivity (75%) was found in the second peak (CA-2). However, when the material in the first pea (CA-1) was treated again with CNBr, more material was produced which eluted in the position of CA-2, indicating that CA-1 contained incompletely cleaved material. The amino terminal residues of CA-1 were proline, aspartic acid, glutamic acid, serine, and glycine. The material in CA-2 gave glutamic acid and serine as amino termini. CA-3 showed glycine and a trace of valine as amino termini.

CA-2 was treated again with dithiothreitol in 6 M guanidine-HCl–0.5 M Tris-HCl (pH 9.0), alkylated with sodium iodoacetate, and rechromatographed on Sephadex G-75 (Figure 1b). Two peaks were obtained: one (CA-4) eluting in the same position as CA-2, and the second (CA-5) eluting later. The material in each peak had both glutamic acid and serine at their NH_2-termini as shown by dansylation.

CA-5 was resolved into two components (CA-6 and CA-7) on DE52 as shown in Figure 1c. CA-6 gave serine as the amino terminus while CA-7 had amino terminal glutamic acid. The peaks were radioactive (33% of the total radioactivity applied to the column was recovered in CA-6 and 34% of the applied radioactivity was recovered in CA-7) indicating that both peptides contained residues labeled with Dnp-N_3.

The results were as follows: CA-1 consists of larger molecular weight CNBr fragments and perhaps some uncleaved material which elutes in the position of CA-1; CA-2 consists of two fragments, CA-6 and CA-7, linked by a disulfide bridge; CA-4 possibly represented unreduced CA-2 since it eluted at the same position from Sephadex G-75; CA-5 contained the same two fragments as CA-2, with the disulfide bridge broken. These fragments, CA-6 and CA-7, could be resolved on DE52.

Characterization of the NH_2-Terminal CNBr Fragment

CA-7. This fragment, obtained from the material in peak CA-5 by anion exchange chromatography on DE52, had the amino acid composition shown in Table I. It contained 34 residues including one residue each of carboxymethyl-cysteine (CM-cysteine) and homoserine. The amino terminal sequence, determined by the dansyl-Edman procedure, was: Glu-Val-Glx-Leu-Glx-Glx- Ser . . . , and was identical with the amino terminal sequence of protein 460 heavy chain. Fragment CA-7 is therefore the amino terminal CNBr fragment of protein 460 heavy chain, contains a Dnp-N_3 modified residue(s), and by comparison to the known sequence of MOPC 173 heavy chain (Bourgois and Fourgereau, 1970) and protein 315 heavy chain (L. Hood, personal communication) would be expected to contain the first heavy chain "hypervariable" region (Wang *et al.*, 1971; Kehoe and Capra, 1971; Capra, 1971).

Characterization of the CNBr Fragment CA-6. The amino acid analysis of CA-6, Table I, showed the presence of one residue each of CM-cysteine and homoserine, and gave a total of approximately 33 residues. The amino terminal sequence, determined by the dansyl-Edman procedure, was: Ser-Lys-Ile-Arg . . . . By comparison to the MOPC 173 heavy chain sequence, CA-6 was thought to contain the second and third heavy chain "hypervariable" regions.[2] The radioactivity associated with CA-6 indicates the presence of a Dnp-N_3 modified residue(s).

Digestion of CNBr Fragment CA-2 with Thermolysin and the Isolation and Characterization of the Labeled Peptides. Because CA-6 and CA-7 could only be obtained in low yields after DE52 chromatography, we decided to isolate small labeled peptides directly from fragment CA-2 and place them in the protein 460 heavy chain sequence by homology with MOPC 173. Thus, CA-2 was digested with thermolysin and subjected to high-voltage electrophoresis at pH 1.9 and pH 6.5. At pH 6.5 all of the radioactivity was located in the neutral region. At pH 1.9, the digest was resolved into three radioactive components (Figure 2) and most of the ninhydrin positive material migrated faster than the radioactivity at pH 1.9.[3] As shown in Figure 2, TH-1 contained approximately 30%: TH-2, 60%: and TH-3, 10% of the total radioactivity. The regions containing the radioactive components were eluted with 30% acetic acid. Both TH-1 and TH-2 showed dansyl-serine and dansyl-glycine as well as a major spot of dansyl-isoleucine. The material in TH-1 and TH-2 was purified further by paper chromatography.

After paper chromatography, TH-1 gave a single radioactive spot (TH-1a) with the amino acid composition: Ile, Thr, Asn, Gly, homoserine (the assignment of Asn was made from the electrophoretic behavior of TH-1: neutral at pH 6.5, positively charged at pH 1.9). The presence of homoserine suggested that this peptide represented the carboxyl-terminus of either CA-6 or CA-7. The dansyl-Edman degradation gave the sequence: Ile-Thr . . . . Additional data beyond the second residue could not be obtained. This may have been due to the fact that the third and fourth residues were Asn-Gly (determined later with the sequenator). When an Asn-Gly sequence is encountered,

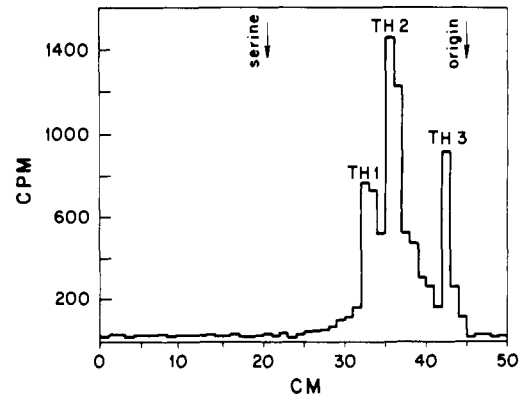

FIGURE 2: The pH 1.9 high voltage electrophoresis of the thermolysin digest of CNBr fragment CA-2; 3000 V; 45 min.

the Edman degradation does not go to completion and the yield of the residues distal to this sequence drops off dramatically due to a rearrangement of an α- to a β-aspartyl linkage (Weber and Konigsberg, 1967; Jörnvall, 1973).

After paper chromatography TH-2 also gave a single radioactive spot (TH-2a) with the composition: Ile, Arg, Ala, Asx, Glx, Thr, Tyr. Again, only the first two residues, Ile-Arg . . . , could be obtained by dansyl-Edman degradation. This may be due to the fact that the third residue, which was subsequently shown to be Dnp-N_3 labeled tyrosine (see following section), is not easily cleaved during the Edman reaction (see Discussion). TH-3 was not further characterized.

Identification and Location of the Dnp-N_3 Labeled Residues and the Sequences of CA-6 and CA-7. CA-7 had previously been shown to be the amino terminal CNBr fragment. The sequence of CA-7 could therefore be obtained by the automated sequencing of Dnp-N_3 labeled protein 460 heavy chain. It was therefore possible to derive the sequence of CA-6 by subjecting the CNBr fragment CA-5 to automated sequencing and subtracting out the residues coming from CA-7. CA-5 was an equimolar mixture of CA-6 and CA-7. Since only 50% of the heavy-chain molecules were labeled by the affinity labeling reagent, both modified and unmodified CA-6 and CA-7 were present in CA-5. The identification and placement of the Dnp-N_3 labeled residues was obtained by automatic sequencing. The amino terminal sequences of protein 460 heavy chain and of fragments CA-6 and CA-7 are given in Table II. The protein 460 heavy chain, partially labeled with Dnp-N_3, was sequenced by P. Barstad and L. Hood (personal communication). They reported the extraction of radioactivity associated with a tyrosine residue into the chlorobutane at step 33. We found that some radioactivity was extracted into the chlorobutane and benzene at each step of the degradation of CA-5, which may have been due to the extraction of labeled peptide into the organic phase or to washing out of the peptide from the cup. However, there was a significant increase in the radioactivity extracted at steps 5 and 33 as shown in Figure 3. The 6444 cpm found in the fifth chlorobutane extract, which was 0.7% of the radioactivity placed in the reaction cup, was six times the background found throughout the chlorobutane wash. The 20174 cpm in the sixth benzene wash was 2.1% of the initial radioactivity and was six times the background found throughout the benzene wash. The 1500 cpm in the 33rd chlorobutane extract was 0.15% of the initial radioactivity and was twice the background. The appearance of radioactivity and the sequence data suggested that tyrosines in position 5 of CA-6 and position 33 of CA-7 were the labeled residues (see Discussion). After 40 cycles of degradation of CA-5, the run was terminated and

[2] Fragment CA-6 is composed of approximately 33 residues. The equivalent region in MOPC 173 heavy chain contains only 26 residues and spans positions 84–109. Thus, it appears that the variable region of protein 460 heavy chain may be seven residues longer than the variable region of MOPC 173 heavy chain.

[3] The mobilities of TH-1 and TH-2 at pH 1.9 relative to serine (Offord, 1966) did not give accurate estimates of the molecular weights of the peptides since they moved too slowly for penta- or heptapeptides with one positive charge. This was probably due to the interaction of the Dnp ring of the labeling reagent with the paper.

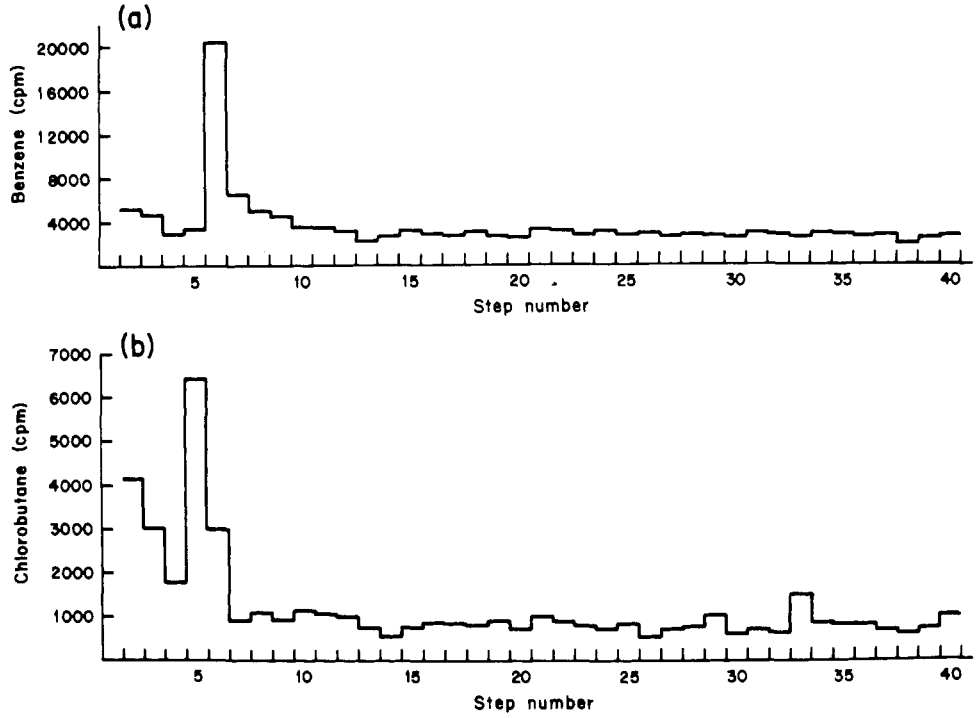

FIGURE 3: Radioactivity present in (a) benzene washes and (b) chlorobutane extractions.

50% of the initial radioactivity remained in the reaction cup; the overall recovery of radioactivity from the cup, chlorobutane extractions, and benzene washes was greater than 75% of the amount originally used.

Discussion

The labeling pattern observed when protein 460 is reacted with Dnp-N_3 differs from the pattern found when Dnp-AD is used. Dnp-AD reacted almost exclusively with the light chain, whereas Dnp-N_3 reacted mainly with the heavy chain (Yoshioka *et al.*, 1973). The light-chain-labeling patterns have been published previously (Hew *et al.*, 1973). The approach used to locate the position of the residues modified with Dnp-N_3 consisted of cleaving the protein 460 heavy chain (which contained a mixture of modified and unmodified molecules) with CNBr, isolating the radioactive fragments, and determining their sequence and the positions of the labeled residues with the protein sequenator. The location of the labeled residues was confirmed by isolation of small labeled peptides obtained by digestion of the CNBr fragment, CA-2, with thermolysin.

The labeled residues were identified as tyrosines and assigned to fragments CA-6 and CA-7 in the following way. (a) Automatic sequencing of Dnp-N_3 labeled protein 460 heavy chain released radioactivity together with PTH-tyrosine at step 33 (P. Barstad and L. Hood, personal communication). (b) Automatic sequencing of CA-5 (a mixture of CA-6 and CA-7) released radioactivity at steps 5 and 33. The radioactivity from the fifth residue must have come from CA-6 since no radioactivity was associated with position 5 of protein 460 heavy chain (CA-7 was identical with the amino terminal CNBr fragment of heavy chain). Position 5 in CA-6 was identified as tyrosine, and by comparison of the sequence around this residue to the sequence in MOPC 173, this tyrosine was assigned to position 88 in the heavy chain sequence. (c) Thermolysin digestion of CNBr fragment CA-2, which consisted of CA-6 and CA-7 linked by a disulfide bridge, gave two major radioactive peptides (TH-1a and TH-2a). These two peptides accounted for 90% of the total radioactivity in the heavy chain.

The data obtained by the automatic sequencing of CNBr fragment CA-5 confirm that TH-2a corresponds to residues 3–10 of fragment CA-6 (tentatively identified by homology with the MOPC 173 heavy chain sequence as residues 86–93), and that TH-1a corresponds to residues 29–34 of the amino terminal CNBr fragment CA-7 and protein 460 heavy chain.

The reaction of Dnp-N_3 with protein 460 heavy chain is confined to two small segments from the variable region since di-

TABLE II: Amino Acid Sequence of the Amino Terminus of Protein 460 Heavy Chain and the Dnp-N_3 Labeled Peptides.

Position No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
Protein 460[a]	Glu	-Val-	Glx	-Leu-	Glx	-Glx-	Ser	-Gly-	Pro	-Ser	-Leu-	Val	-Lys-	Pro	-Ser	-Glx	-Thr	-Leu	-Ser	-Leu	-Thr	(Cys)	Ser	-Val	-Thr-
CA-7	Glu	-Val-	Glx	-Leu-	Glx	-Glx-	Ser	-Gly-	Pro	-Ser	-Leu-	Val			Ser	-Glx	-Thr	-Leu	-Ser	-Leu	-Thr			Val	-Thr
CA-6	Ser	-Lys-	Ile	-Arg-	Tyr	-Ala	-Glx	-Asx	-Thr	-Tyr	-Tyr	-Asx													

Position no.	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40
Protein 460	Gly	-Ser	-Asx	-Ile	-Thr	-Asn	-Gly	-Tyr	-Met	-Asx	-Trp	-Ile	(Arg)	Lys	-Phe
CA-7				Ile				Tyr							
CA-6															

[a] P. Barstad and L. Hood (personal communication).

gestion of CA-2 with thermolysin produces only two radioactive peptides (TH-1a and TH-2a). These peptides were placed in the protein 460 heavy chain sequence by their compositions, amino terminal sequences, homology to MOPC 173 and by information derived from the partial sequencing of protein 460 heavy chain.

The protein sequenator was used to determine the amino terminal sequences of protein 460 heavy chain, CA-6 and CA-7. During the automatic sequencing of CA-6 and CA-7, 50% of the radioactivity remained in the reaction cup after 40 cycles, although the radioactivity which was extracted into the chlorobutane did permit the identification of the labeled residues. The fact that more radioactivity was not extracted into the chlorobutane washes in the steps following 5 and 33 indicated that the small percentage of extractable counts was not due to a slower rate of cleavage for the labeled residue.

The Asn-Gly sequence at positions 31 and 32 of protein 460 heavy chain and CA-7 may contribute to the small amount of radioactivity extracted at step 33. If most of the α-peptide bond between the Asn and Gly residues had rearranged to the β-aspartyl linkage, the Edman degradation would not have proceeded very well beyond this residue and therefore only a small amount of the tyrosine at position 33 would have been released. The fact that the manual dansyl-Edman degradation of the thermolysin peptide TH-1a could not be continued beyond the second residue if consistent with this explanation.

Approximately 60% of the radioactivity incorporated into heavy chain was associated with the thermolysin peptide TH-2a. The Edman degradation of TH-2a also failed after the second residue. The third residue was subsequently shown to be a labeled tyrosine (position 88 in heavy chain). The automatic sequencing of CA-6 released a small, but significant, amount of radioactivity associated with this labeled tyrosine. A possible explanation for these results is that there may be two reaction products of Dnp-N_3 with tyrosine-88 (also possibly with tyrosine-33). One product would be subject to acid cleavage while the other product would not be cleaved by treatment with trifluoroacetic acid or heptafluorobutyric acid and it is this product that would block the Edman degradation. The results suggest that most of the reaction product of Dnp-N_3 with tyrosine-88 (in CA-6) was the acid-resistant product thus resulting in the retention of the majority of the radioactivity in the reaction cup.

Molecular models of *N*-phenylthiocarbamyl-L-tyrosyl-2,5-azobis(2,4-dinitrobenzene), the ortho-substituted Dnp-N_3 derivative of phenyl isothiocyanate tyrosine, provide no evidence suggesting steric interference with ring closure to the thiazolinone. The possibility therefore exists that the nitrene has inserted into the α carbon of tyrosine. Since the phenylthiocarbamyl moiety provides a convenient amino terminal marker, the modified tyrosine should be detectable by high-resolution mass spectroscopy. Tyrosine cleaves at the benzylic bond with charge retention on either side (Vetter, 1972) and the presence of a major benzyl fragment ion of *m/e* 91 would indicate an unsubstituted tyrosine ring. Experiments to check the structure of the reaction products in this way are planned. There is also an indication that modification of tyrosine-33 by Dnp-N_3 reduces the effectiveness of the cyanogen bromide cleavage of methionine-34 since we found a reciprocal relationship between the degree of substitution by Dnp-N_3 and the extent of cleavage at the methionine residue. Thus, a chemically modified amino acid residue may not only affect changes in the rate of proteolytic enzyme digestion close to the modified residue (Johansen *et al.*, 1958; Peterson *et al.*, 1974), but the presence of a chemically modified residue also may alter the rate of chemical cleavage.

The overall affinity labeling pattern of protein 460 as described in this and the two preceding publications (Yoshioka *et al.*, 1973; Hew *et al.*, 1973) and its significance with respect to the conclusions that can be drawn from the affinity labeling of immunoglobulins in general will be discussed in the accompanying paper (Richards *et al.*, 1974).

References

Bourgois, A., and Fougereau, M. (1970), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 8,* 265.

Capra, J. D. (1971), *Nature (London), New Biol. 230,* 61.

Converse, C. A., and Richards, F. F. (1969), *Biochemistry 8,* 4431.

Gray, W. R. (1967), *Methods Enzymol. 11,* 139.

Gray, W. R. (1972), *Methods Enzymol. 25,* 121.

Gross, E. (1967), *Methods Enzymol. 11,* 238.

Hew, C.-L., Lifter, J., Yoshioka, M., Richards, F. F., and Konigsberg, W. H. (1973), *Biochemistry 12,* 4679.

Inglis, A. S., Nicholls, P. W., and Roxburgh, C. M. (1970), *Aust. J. Biol. Sci. 24,* 1247.

Johansen, P. G., Marshall, R. D., and Neuberger, A. (1958), *Nature (London) 181,* 1345.

Jörnvall, H. (1973), *Proc. Int. Congr. Biochem., 9th,* 454.

Kehoe, J. M., and Capra, J. D. (1971), *Proc. Nat. Acad. Sci. U. S. 68,* 2019.

Konigsberg, W. H. (1972), *Methods Enzymol. 25,* 185.

Matsubara, H. (1966), *Biochem. Biophys. Res. Commun. 24,* 427.

Matsubara, H., Singer, A., Sasaki, R., and Jukes, T. H. (1965), *Biochem. Biophys. Res. Commun. 21,* 242.

Offord, R. E. (1966), *Nature (London) 211,* 591.

Peterson, R. G., Richards, F. F., and Handschumacher, R. (1974), *Biochemistry,* submitted for publication.

Pisano, J., Bronzert, T. J., and Brewer, H. B., Jr., (1972), *Anal. Biochem. 54,* 430.

Richards, F. F., Lifter, J., Hew, C.-L., Yoshioka, M., and Konigsberg, W. H. (1974), *Biochemistry 13,* 0000.

Smithies, O., Gibson, D., Fanning, E. M., Goodfliesh, R. M., Gilman, J. G., and Ballantyne, D. C. (1971), *Biochemistry 10,* 4912.

Vetter, W. (1972), *in* Biochemical Application of Mass Spectrometry, Waller, G. R., Ed., New York, N. Y., Wiley-Interscience, p 387.

Wang, A. C., Fudenberg, H. H., and Pink, J. R. L. (1971), *Proc. Nat. Acad. Sci. U. S. 68,* 1143.

Weber, K., and Konigsberg, W. H. (1967), *J. Biochem. 242,* 3563.

Weiner, A. M., Platt, T., and Weber, K. (1972), *J. Biochem. 247,* 3242.

Yoshioka, M., Lifter, J., Hew, C.-L., Converse, C. A., Armstrong, M. Y. K., Konigsberg, W. H., and Richards, F. F. (1973), *Biochemistry 12,* 4685.