The Antibody Binding Site

LABELLING OF A SPECIFIC ANTIBODY AGAINST THE PHOTO-PRECURSOR OF AN ARYL NITRENE

By G. W. J. FLEET,*† J. R. KNOWLES† and R. R. PORTER*

*Medical Research Council Immunochemistry Unit, Department of Biochemistry, University of Oxford, Oxford OX1 3QU, and †The Dyson Perrins Laboratory, University of Oxford, Oxford OX1 3QY, U.K.

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The isolation of specific rabbit antibodies for the haptenic group 4-azido-2-nitrophenyl, is described. These antibodies bind 1.8–2.0 mol of hapten [ϵ -(4-azido-2-nitrophenyl)-L-lysine]/mol with an association constant of nearly 10^7 M^{-1} at 4°C. On photolysis of the antibody-hapten complex, resulting in the formation of an aryl nitrene at the binding site, hapten was covalently bound to the antibody, and the antibody binding site was blocked. The ratio of labelling of heavy- and light-chains was 2.5:1. Two small peptides were isolated from digests of labelled heavy-chain, indicating that some 13% of the label in the antibody was attached to cysteine-92 and to alanine-93. These residues are adjacent to the major hypervariable region in rabbit heavy-chain (residues 95–105).

Amino acid residues that may be involved directly in the catalytic action of an enzyme can often be labelled with an active-site-directed irreversible inhibitor (Baker, 1967). Such inhibitors must (a) contain the structural elements required for specific binding at the active site, (b) not react rapidly with water, and (c) not react too vigorously with the protein amino acids (otherwise significant amounts of random labelling will occur). The rationale is that an amino acid at the active site will react preferentially with the relatively high local concentration of reagent present there. This approach, by the nature of the chemically reactive groups in proteins, tends to search out nucleophiles at active sites (Cohen, 1968; Vallee & Riordan, 1969). This restriction to the six or so nucleophilic amino acids is not a serious limitation in enzymology, since here one is particularly interested in the identity of these groups, which often have enhanced reactivities.

There are two reasons why the approach for enzyme labelling is inappropriate for antibodies. First, antibodies do not do anything in chemical terms (i.e. they do not catalyse chemical transformations, but merely bind their ligand), and there is no reason to *a priori* expect reactive nucleophiles at the binding sites of antibodies. The active-site-directed inhibitor technique relies on the high local concentration of reagent at the binding site, and this method may produce misleading labelling patterns, in which the nearest (in space, though not of course necessarily in sequence) reasonably accessible nucleophile will be labelled. The second reason is that the enzymological

approach fails to exploit two important features of antibody molecules. First, dissociation constants of antibody-hapten complexes can be very small, which means that the protein can essentially be titrated with the reagent to obtain a stoichiometric complex, and non-specific reaction by excess of reagent not in the binding site is minimized. Secondly, the specific antibody molecules are selected by the immunized animal to fit the reagent, and one is not constrained in synthetic terms by the structural restrictions of, for instance, an enzyme's natural substrate. These considerations mean that the design of reagents for antibodies should be different from that for enzymes: a completely unselective reagent is required which reacts even with hydrocarbon amino acid side-chains at the binding site, and which exploits the fact that the protein is made to fit the reagent rather than the converse.

Previous attempts to label the binding sites of antibodies have generally used either a difference-labelling technique (see, e.g., Pressman & Roholt, 1961), or the affinity-labelling method analogous to that used in enzymology (see, e.g., Singer & Doolittle, 1966). The former approach is limited by the facts that (a) only chemically functional residues can be investigated; (b) no account can be taken of differential reactivity of particular residues in different environments on the protein surface; (c) the first reaction of reagent with the complex may affect the conformation of the protein; and (d) any protein conformational change consequent on hapten removal may expose residues other than those at the

binding site. The latter technique is not ideal in that (a) only a very limited range of amino acids is susceptible to attack by the reagent, (b) the method relies on cross-reaction, the reagent being a structural analogue of the hapten.

Haimovich *et al.* (1970) have used two electrophilic affinity labels to probe the binding site of the mouse myeloma protein MOPC 315. This kind of 'mapping' approach will prove useful in defining parts of the antibody near to the specificity locus, but is still open to the objection that only a limited number of nucleophilic amino acids can be labelled. Any approach based on a reagent added directly to an aqueous solution of antibody is bound to be severely limited in that water is more reactive than very many of the amino acid side-chains. So despite the advantage of a higher local concentration which the structural analogue of a hapten or substrate possesses, it is not enough to limit the chemical range of possible reagents to those that have half-lives in neutral aqueous solution of longer than a few minutes. As has been pointed out above, a reagent is required capable of attacking even C-H bonds at the specificity site. Clearly such a species cannot be added externally to aqueous solution of protein, and must be generated from a stable precursor *in situ*.

The only chemical intermediates capable of C-H bond insertion are carbenes, nitrenes, and other such reactive species. These can be generated photochemically, though for present purposes, the precursor must be (a) chemically inert (if it is not, then antibodies will be produced primarily to the decomposition products of the reagent precursor), (b) photolysable to a species that does not rearrange intramolecularly to a less reactive entity, and (c) susceptible to photolysis at wavelengths well clear of protein absorption (to avoid photo-oxidative damage to the protein on irradiation).

There are no really stable photo-precursors of carbenes, though as nitrene precursors, aryl azides have the required chemical stability. We report here the preparation and use of an aryl azide as a haptenic group, the isolation of the antibody, and the identification of two labelled peptides from the heavy-chain of the antibody-radioactive-hapten complex after irradiation. A preliminary report of some of this work has appeared (Fleet *et al.*, 1969).

Experimental

Materials

Iodoacetic acid was recrystallized from diethyl ether, washed with light petroleum (b.p. 60–80°C) and stored in the dark at 4°C. Iodo[2-¹⁴C]acetic acid and L-[4,5-³H]lysine monohydrochloride were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Bovine γ -globulin (Cohn fraction II) was

obtained from Mann Research Laboratories Inc., New York, N.Y., U.S.A. It was dialysed against 3mM-EDTA–0.1M-NH₄HCO₃ buffer, pH 9.0, and then freeze-dried. Rabbit IgG* was obtained from pooled rabbit serum by the method of Prahl & Porter (1968). Human serum albumin was a gift from the Lister Institute Blood Production Unit. It was dialysed against 3mM-EDTA–0.1M-NH₄HCO₃ buffer, pH 9, and then freeze-dried. Cyanogen bromide was obtained from Eastman Organic Chemicals, Rochester, N.Y., U.S.A. Trypsin, treated with chloromethyl L-(1-tosylamido-2-phenyl)ethyl ketone, and carboxypeptidase B were obtained from Worthington Biochemical Corp., Freehold, N.J., U.S.A.

4-Fluoro-3-nitrophenyl azide. Crude (90% pure) 4-fluoro-3-nitroaniline (5.0g) (Koch-Light Laboratories, Ltd., Colnbrook, Bucks., U.K.) was dissolved by warming in a mixture of conc. HCl (30ml) and water (5ml). The solution was filtered and cooled. NaNO₂ (2.4g) in water (5ml) was added slowly while the temperature was maintained between –20°C and –15°C by using an external bath of acetone–solid CO₂. After the addition was complete, the reaction mixture was stirred for 10min and then rapidly filtered into a flask at –20°C. NaN₃ (2.2g) in water (8ml) was added at –20°C; during the addition N₂ was evolved and an orange precipitate separated. This solid was collected by filtration and washed with ice-cold water. Recrystallization from light petroleum (b.p. 40–60°C) gave 4-fluoro-3-nitrophenyl azide (4.5g; yield 75%) as straw-coloured needles, m.p. 52°C. The azide is soluble in ethanol, ether, acetone and carbon tetrachloride. The dry azide is explosive and large quantities should be handled with care. It may be stored safely at 4°C, moistened with water (Found: C, 39.7; H, 1.85; N, 31.0; F, 10.9. C₆H₃N₄O₂F requires: C, 39.6; H, 1.65; N, 30.8; F, 10.4%), $\nu_{\max}$. 2130, 2090cm^{–1} (N₃), $\lambda_{\max}$. 242nm (in ethanol). [*m/e* (% intensities relative to 108 peak): 182 (53), 154 (44), 109 (20), 108 (100), 82 (30), 81 (41); metastable peaks: 130.6, 75.6.]

4-Azido-N-methyl-2-nitroaniline. 4-Fluoro-3-nitrophenyl azide (200mg) was dissolved in ethanol (5ml). To this solution 3ml of aq. 30% (w/v) methylamine was added. A red precipitate appeared almost immediately. The reaction mixture was left at room temperature for 1h and the product was then collected by filtration and washed with water until free of methylamine. Recrystallization from ethanol gave 4-azido-N-methyl-2-nitroaniline (150mg, yield 73%) as red needles, m.p. 122–123°C (Found: C, 43.7; H, 3.54; N, 36.6. C₇H₇N₅O₂ requires: C, 43.5; H, 3.60; N, 36.3%), $\lambda_{\max}$. 220, 258 and 458nm (molar extinction coefficients: 7700, 26750 and 4640) in

* Abbreviations: IgG, immunoglobulin G; Cys-CH₂CO₂H, carboxymethylcysteine; Nap, 4-azido-2-nitrophenyl.

Table 1. *Amino acid analysis of the antigen preparation Nap-bovine γ -globulin*

For experimental details see the text.

Ratio of	Preparation		
	Untreated	Batch 1	Batch 2
Arg:His	2.48	2.26	2.27
Lys:His	3.45	1.30	0.085
Lys:Arg	1.39	0.574	0.037

ethanol, $\nu_{\max}$. 3400 (NH), 2918 and 2945 (CH), 2115 and 2078 (N_2) cm^{-1} .

The relative rate of reaction of 2,4-dinitrofluorobenzene and 4-fluoro-3-nitrophenyl azide with a large excess of aqueous methylamine was followed by measuring the E_{460} and was found to be approx. $10^3:1$.

Preparation of the antigen, Nap-bovine γ -globulin. Bovine γ -globulin (1g) was dissolved in 0.0125M-sodium borate buffer, pH9.8 (100ml) and was treated with a solution of 4-fluoro-3-nitrophenyl azide (0.4g) in ethanol (10ml); the resulting reaction mixture, protected from the light, was stirred at about 40°C for 3h. A sample was removed for analysis (batch 1). Further azide (0.5g) in ethanol (5ml) was then added to the reaction mixture which was stirred overnight (batch 2). The red reaction mixture was then filtered. The filtrate was dialysed against several changes of water for 3 days, and was then filtered and freeze-dried to give the antigen, Nap-bovine γ -globulin.

The number of mol of Nap groups per mol of γ -globulin was determined by amino acid analyses (see Table 1), by measurement of the E_{460} , and by measurement of N_2 evolution during irradiation.

Amino acid analysis indicates that 60% of the lysine residues were modified in batch 1 and 97% (i.e. about 60 residues) in batch 2. Lysine was the only amino acid modified. For the spectroscopic determinations of the number of Nap groups, the E_{280} and E_{460} were determined. The extinction coefficients of 4-azido-*N*-methyl-2-nitroaniline at these two wavelengths were used for the calculation. Agreement with the amino acid analysis was close.

The volume of N_2 evolved during irradiation of a solution of Nap-bovine γ -globulin at 35°C by a Mazda 125W MB/V pearl-glass lamp immersed in a solution of $NaNO_2$ (to absorb any radiation of shorter wavelength than 400nm) was measured in a Warburg apparatus. The evolution was quantitative and was completed within 18h. The amount of N_2 evolved corresponded closely to the number of Nap groups present, as determined by the other two methods. A solution of antigen treated similarly but protected from the irradiation did not yield any N_2 . This demonstrated that the Nap group was thermally

stable under physiological conditions and was smoothly photolysed on irradiation at wavelengths above 400nm.

Nap-human serum albumin. Nap-human serum albumin was prepared by a similar method to that for Nap-bovine γ -globulin. Some protein came out of solution during the modification, and this was discarded. Approx. 30% of the lysine residues were modified.

4-Azido-2-nitrophenol. This was prepared by the method of Forster & Fierz (1907).

N-4-Azido-2-nitrophenylglycine. Glycine (0.5g) and Na_2CO_3 (1.2g) were dissolved in water (12ml). The 4-fluoro-3-nitrophenyl azide (1g) was added as a solid and sufficient ethanol (approx. 15ml) to form a homogeneous solution was added. The mixture was refluxed for 4h in the dark. The reaction mixture was cooled, diluted with water (15ml), filtered, and the filtrate was extracted with ether (2×50 ml). The aqueous layer was poured into dilute HCl (40ml) and again extracted with ether (3×100 ml). The combined ethereal extracts were dried over anhydrous $MgSO_4$ and then evaporated to dryness. The resulting residue was crystallized from ethanol-water to give *N-4-azido-2-nitrophenylglycine* (1.0g, 77%) as red needles, m.p. 173–174°C. (Found: C, 40.9; H, 3.03; N, 29.7. $C_8H_7N_5O_4$ requires: C, 40.5; H, 2.95; N, 29.5%). $\lambda_{\max}$. 261 and 459nm (molar extinction coefficients: 28300 and 4800) in ethanol. $\nu_{\max}$. (chloroform) 2210 and 2082 cm^{-1} (N_2).

ϵ -(4-Azido-2-nitrophenyl)-L-lysine and ϵ -(4-azido-2-nitrophenyl)-L-[4,5- 3H]lysine. L-Lysine monohydrochloride (25mg) in 0.1M- Na_2CO_3 (5ml) was added to 4-fluoro-3-nitrophenyl azide (50mg) in ethanol (5ml) and the reaction mixture was incubated at 60°C overnight. The solution was then applied to silica gel G plates (20cm $\times$ 20cm) and the plates were developed in butanone saturated with water for approx. 2h. Three red products were obtained. The two slowest-running components were only just separated and care was necessary to avoid loss of resolution caused by overloading of the plate. The compounds were eluted from the silica gel with water. This separation could also be performed by using the same solvent system on Whatman 3MM paper. The yields of the compounds were estimated spectroscopically by assuming the molar extinction coefficient, ϵ , to be 4800 per Nap group at 460nm by analogy with Nap-glycine. Each of the three compounds had u.v.-absorption maxima at 260 (± 4)nm and 478 (± 3)nm. The ratio of the extinction coefficients at 260 and 460nm is given in Table 2. The volumes and yields of N_2 evolved when the compounds were irradiated as described for Nap-bovine γ -globulin for 12h, were measured in a Warburg apparatus, and are given in Table 2. From this, it is clear that the fastest-running compound is $\alpha\epsilon$ -diNap-L-lysine, and that the other two components

Table 2. *Products from the reaction of L-lysine with 4-fluoro-3-nitrophenyl azide*

For experimental details see the text.

Compound	Yield (%) based on lysine	Ratio of molar extinction coefficients	mol of N ₂	mol of Nap†
		$\epsilon_{260}:\epsilon_{460}$	mol of derivative	mol of lysine
α -Nap-lysine	9	5.8	0.79	1.13
$\alpha\epsilon$ -diNap-lysine*	14	6.1	1.82	1.91
ϵ -Nap-lysine	18	6.0	0.88	0.93
Nap-glycine	—	5.9	0.92	—

* $\alpha\epsilon$ -diNap-lysine was assumed to have $\epsilon=9600$ at 460 nm.† This ratio was calculated from the radioactivity of the product of reaction of [³H]lysine.

are the α - and the ϵ -monoNap derivatives. These two materials were distinguished as follows. α -t-Butyloxycarbonyl-L-lysine (50mg) in 0.1M-Na₂CO₃ (2ml) was treated with 4-fluoro-3-nitrophenyl azide (50mg) in ethanol (2ml) and the reaction mixture was refluxed for 2h. The solution was then cooled and extracted into ether. The aqueous layer was acidified with dilute HCl and rapidly extracted with ether. The combined ether extracts were dried over anhydrous MgSO₄ and then evaporated to give a red oil, that did not crystallize. The red oil was treated with trifluoroacetic acid (5ml) at room temperature and the product was subjected to t.l.c. on silica gel as described above. From this, it was clear that, in the preparation with free lysine, the faster-running of the two mono-substituted derivatives is ϵ -Nap-lysine. The two mono-substituted derivatives migrated as expected on paper electrophoresis at pH8.9.

The ϵ -Nap-L-[³H]lysine was prepared directly from L-[4,5-³H]₂lysine monohydrochloride (209mCi/mmol; 1.18×10^8 c.p.m./ μ mol) by using 4-fluoro-3-nitrophenyl azide. The molar ratio of L-[³H]lysine to Nap groups (estimated from the E_{460}) in the products is given in Table 2.

Methods

U.v. spectra were measured with either a Unicam SP.800 recording spectrophotometer or a Cary 14 instrument. Fixed-wavelength measurements were made with a Unicam SP.500 instrument.

I.r. spectra were recorded with a Perkin-Elmer 237 recording spectrophotometer.

Mass spectra were determined by Dr. R. Aplin in the Dyson Perrins Laboratory with an A.E.I. MS9 instrument.

Scintillation counting was performed with a Beckman LS100 liquid-scintillation spectrophotometer. Kinard's (1957) scintillation fluid was used.

Amino acid analyses were done with a Beckman-Spinco model 120 instrument, or a Locarte analyser, by the procedures of Spackman *et al.* (1958).

Vertical paper electrophoresis was done by the method of Michl (1951), as modified by Katz *et al.* (1959).

Melting points were determined on a Kofler block and are uncorrected.

Microanalyses were performed by Dr. Strauss, in the Dyson Perrins Laboratory.

Radioautography was performed as described by O'Donnell *et al.* (1970).

Immunization. The 12 Dutch Grey rabbits were given a primary subcutaneous injection of Nap-bovine γ -globulin (5mg) in Freund's complete adjuvant. A booster injection was given intravenously 5 weeks later either with 1 mg of antigen in adjuvant or with 1 mg of alum-precipitated antigen; the second method gave a slightly better response. The animals were bled at intervals over a period of 4 months, with intermediate booster injections when assays of the sera indicated that they were needed. The antibody response was 400–800 μ g/ml of antibody precipitable with Nap-human serum albumin.

Isolation of antibodies. Anti-Nap antibody was isolated from pooled antisera by the method of Eisen (1964a, 1967). The antiserum was precipitated with Nap-human serum albumin and the resulting precipitate was stirred in a saturated solution of 4-azido-2-nitrophenol in 0.09M-NaHCO₃–0.01M-Na₂CO₃ buffer (pH9)–10% (v/v) dimethylformamide at 37°C overnight. The suspension was centrifuged and the supernatant was passed down a column of DEAE-cellulose packed on top of Dowex 1 (X8), equilibrated with 30mM-sodium phosphate buffer, pH7.4. This treatment removed the antigen and the hapten. The isolated anti-Nap antibody showed no E_{460} and gave a single slow-moving band on electrophoresis on cellulose acetate, a single symmetrical peak (s_{20} 6.3S) on ultracentrifugation and a single line in double-diffusion against goat anti-(whole rabbit serum). The affinity constant of the anti-Nap antibody for ϵ -Nap-[³H]lysine as measured by equilibrium dialysis (Eisen, 1964b) ranged from 5.3×10^6 to 1.0×10^7 M⁻¹ at 4°C.

Separation of chains. Antibody was mildly reduced, alkylated and separated into heavy and light chains by the method of Prahl & Porter (1968). The amount of heavy or light chain in solution was estimated by assuming $E_{280}^{0.1\%}$ 1.4 for a 10mm light-path (Wilkinson, 1967).

Digestion of heavy-chain. The fragment C-1 from heavy chain was obtained from a cyanogen bromide digest (Cebra *et al.*, 1968) after it had been fractionated on Sephadex G-100 (Fruchter *et al.*, 1970). This fragment was completely reduced and carboxymethylated with iodo[^{14}C]acetic acid, as described by O'Donnell *et al.* (1970) and Frangione & Milstein (1968).

Results

Photolabelling experiments

Anti-Nap antibody (3ml of a 3mg/ml solution in 0.03M-sodium phosphate buffer, pH7.4) was incubated with a fivefold molar excess of radioactive hapten (ϵ -Nap-[^3H]lysine) at 4°C for 2h in the dark. The mixture was then fractionated on a Sephadex-G-25 column equilibrated with 0.1M- ϵ -Nap-[^3H]lysine in 0.03M-sodium phosphate buffer, pH7.4, and eluted with the same buffer. The protein fraction was eluted at the void volume together with 1.8–2.0mol of hapten/mol of antibody. The remainder of the hapten was eluted 3 void volumes later. The antibody-hapten complex was then irradiated at 4°C for 18h by two Mazda 125W MB/V pearl-glass lamps immersed in a solution of NaNO_2 which absorbed radiation of wavelengths shorter than 400nm.

'Normal' IgG (2ml of a 20mg/ml solution) was added as carrier to a portion of the resulting reaction mixture and the protein was precipitated with 20% (w/v) trichloroacetic acid. The precipitate was washed with 20% (w/v) trichloroacetic acid and then dissolved in 0.1M-NaOH and the radioactivity in this solution determined. This demonstrated that 1.1–1.3mol of [^3H]lysine was covalently bound/mol of protein. (Lower values have been obtained in more recent experiments: see the Discussion section.) When the affinity constant of the labelled antibodies was re-measured, it was found to be too low to obtain a satisfactory value. (If normal rabbit IgG was used in the place of specific antibody, less than 0.1mol of [^3H]lysine was bound by the protein after gel filtration, and subsequent irradiation resulted in 0.04mol of [^3H]lysine/mol of IgG.) Similar results were obtained if the antibody was incubated with 2.2mol of ϵ -Nap-lysine/mol. The antibody-hapten conjugate after gel filtration contained 1.9mol of hapten/mol of protein. If the anti-Nap antibody was incubated with 2.2mol of radioactive hapten/mol and then irradiated without gel filtration to remove excess of hapten, 1.2–1.3mol of [^3H]lysine/mol of protein was covalently bound.

In the large-scale experiment, anti-Nap antibody was photolabelled with ^3H -labelled hapten (1.62×10^7 c.p.m./ μmol) in four portions. The details of one run are as follows. Anti-Nap antibody (196mg; 1.33 μmol) in 0.03M-sodium phosphate buffer, pH7.4 (18ml), was incubated with ^3H -labelled hapten (4.8×10^7 c.p.m.; 2.93 μmol) in the phosphate buffer (3ml) at 4°C for 2h in the dark. The mixture was then irradiated for 24h as described above and was then dialysed against 0.05M- NH_4HCO_3 solution. After exhaustive dialysis the protein solution contained 2.9×10^7 c.p.m., i.e. 1.35mol of [^3H]lysine/mol. The solution was then freeze-dried. In all, anti-Nap antibody (715mg; 4.77 μmol) was labelled with ϵ -Nap-[^3H]lysine (1.03×10^8 c.p.m.; 6.35 μmol), corresponding to 1.28mol of [^3H]lysine/mol of antibody.

Isolation of labelled peptides

Separation of the labelled anti-Nap antibody into heavy and light chains yielded 424mg (75% of recovered protein) of heavy chain containing 6.3×10^7 c.p.m., and 145mg (25% of recovered protein) of light chain containing 1.76×10^7 c.p.m. (see Fig. 1). The heavy-chain labelling corresponds to 0.45mol of Nap groups/mol, and that of the light chain to 0.18mol of Nap groups/mol. Thus the separated

Fig. 1. Fractionation of heavy and light chains of anti-Nap rabbit antibodies labelled with ϵ -Nap-[^3H]lysine

Fractionation was done on a column (110cm $\times$ 4cm) of Sephadex G-100 in 1M-propionic acid. ●, E_{280} ; ○, ^3H radioactivity (c.p.m./25 μl of eluate). The fraction size was 7.5ml.

chains contain $(0.45 \times 0.18) \times 2 = 1.28$ mol of Nap groups/mol of antibody.

The heavy-chain (413mg) was then digested with

cyanogen bromide, and gave fragment C-1 on fractionation (see Fig. 2). The yield of fragment C-1 was 139mg, containing 3.8×10^7 c.p.m. (73% of the

Fig. 2. Fractionation of a cyanogen bromide digest of rabbit anti-Nap heavy chain labelled with ϵ -Nap-[^3H]lysine

Fractionation was done on a column (155cm $\times$ 3cm) of Sephadex G-100 in 6M-urea–0.2M-sodium formate, pH 3.2. $\bullet$, E_{280} ; $\circ$, ^3H radioactivity (c.p.m./25 μl of eluate). The fraction size was 5 ml. The C-1 fraction taken is shown by the arrow.

Fig. 3. Elution diagram of a tryptic digest of the C-1 fragment from anti-Nap antibody heavy chain, labelled with ϵ -Nap-[^3H]lysine (treated with iodo[2- ^{14}C]acetate)

Fractionation was done on a column (160cm $\times$ 3cm) of Sephadex G-50 in 0.05M- NH_4HCO_3 . Δ , E_{280} ; $\circ$, ^3H ; $\bullet$, ^{14}C radioactivity (c.p.m./20 μl of eluate). The fraction size was 5 ml. Peak A (fractions 80–93) and peak B (fractions 52–65) are indicated.

total radioactivity applied to the column). This corresponds to 0.42 mol of Nap groups/mol of fragment C-1.

After total reduction and carboxymethylation with iodo[2- ^{14}C]acetic acid, and tryptic digestion, the mixture was fractionated on Sephadex G-50 (O'Donnell *et al.*, 1970; Frangione & Milstein, 1968) (see Fig. 3).

The total ^3H radioactivity eluted was 3.2×10^7 c.p.m. (84% of the ^3H applied to the column). There were two major peaks containing both ^3H and ^{14}C . Peak A (fractions 80–93) contained 38% of the eluted ^3H (1.2×10^7 c.p.m.) and ^{14}C (2.8×10^5 c.p.m.) and contained peptides containing three to five amino acid residues. Peak B (fractions 51–65) contained 53% of the eluted ^3H (1.7×10^7 c.p.m.) and ^{14}C (4.2×10^5 c.p.m.) and consisted of peptides containing approx. 30 residues. Further purification of these peptides was attempted, but no pure peptides were isolated. Of the eluted ^3H , 91% was contained in peaks A and B.

The radioactive peptides in peak A were separated by paper electrophoresis on Whatman 3MM paper in pyridine–acetic acid–water (1:10:289, by vol.) at pH 3.5 (Katz *et al.*, 1959) at 3000 V for 1 h. Fig. 4 shows the result of one such run. A thin strip from the side of the paper was cut off and stained with ninhydrin; the position of ^{14}C peptides was determined by radioautography. The peptides were eluted from the paper in 70–75% yield by the method of Edstrom (1968). One peptide, A(i), was basic,

Fig. 4. Elution diagram of electrophoretogram (pH 3.5, 3000 V) of peak A obtained from tryptic digest of labelled C-1 fragment

(a) ^3H and (b) ^{14}C radioactivity (c.p.m. eluted from electrophoretogram strips). Peptide A(i) (12–14 cm towards cathode) and peptide A(ii) (3–4 cm towards cathode) are indicated.

whereas the other peptide, A(ii), was neutral. Peptide A(i) contained 3.1×10^6 c.p.m. of ^3H and no ^{14}C . Peptide A(ii) contained 2.1×10^6 c.p.m. of ^3H and 5.0×10^4 c.p.m. of ^{14}C . The total ^3H radioactivity eluted (5.2×10^6 c.p.m.) was approx. 42% of the ^3H applied as peak A.

Investigation of peptide A(i)

A sample of peptide A(i) containing 2.1×10^5 c.p.m. of ^3H (i.e. $0.013 \mu\text{mol}$ of label) was hydrolysed and the resulting amino acids were analysed; arginine ($0.0130 \mu\text{mol}$) and alanine ($0.0152 \mu\text{mol}$) were the only amino acids present.

A further sample of peptide A(i) containing 1.6×10^6 c.p.m. of ^3H was incubated with carboxypeptidase B in 0.02 M-tris–HCl buffer, pH 8.0, at 37°C overnight. Acetic acid (1 ml) was then added and the reaction mixture was freeze-dried. The product was subjected to paper electrophoresis at pH 3.5, with free arginine as a marker. A thin strip of the paper was cut off and stained. There were two ninhydrin-positive spots. The faster-running spot ran with free arginine, and this also gave a positive Sakaguchi reaction. This demonstrated C-terminal arginine in peptide A(i). The slower-running spot was eluted from the paper and found to contain ^3H . It was freeze-dried, hydrolysed and subjected to amino acid analysis, and showed only the presence of alanine. The sequence of peptide A(i) was therefore (X,Ala)-Arg. The ^3H -labelled component, X, of this peptide is taken to be an amino acid residue modified by the nitrene from ϵ -Nap-lysine. If the nitrene has, as expected, inserted into a C–H bond, then the N-alkyl-aniline product will be stable to the acid hydrolysis before amino acid analysis. The ^3H -labelled component was eluted from the analyser only during the column regeneration with NaOH.

Attempts were made to prepare volatile derivatives of peptides A(i) and A(ii) (see below) for examination in the mass spectrometer in the hope of determining the identity of X. Accordingly, peptide A(i) was treated with carboxypeptidase B, and the slower-running peptide was treated with diethyl pyrocarbonate followed by diazomethane (Kamerling *et al.*, 1968). Unfortunately no peaks were observed in the mass spectrum. More material will be needed for such determinations to be made.

Investigation of peptide A(ii)

A sample of peptide A(ii), containing 2.04×10^5 c.p.m. of ^3H (i.e. $0.0126 \mu\text{mol}$ of label) and 4.7×10^3 c.p.m. of ^{14}C , was hydrolysed and shown by amino acid analysis to contain arginine ($0.0136 \mu\text{mol}$), phenylalanine ($0.0165 \mu\text{mol}$) and carboxymethylcysteine ($0.0120 \mu\text{mol}$) together with smaller amounts

of aspartic acid ($0.0021\ \mu\text{mol}$), serine ($0.0053\ \mu\text{mol}$) and valine ($0.0048\ \mu\text{mol}$).

A sample of peptide A(ii) containing 2.6×10^5 c.p.m. of ^3H was taken for *N*-terminal amino acid identification, by the dansyl method of Gray (1967), and phenylalanine was found to be the *N*-terminal residue.

A further sample of peptide A(ii) containing 1.1×10^6 c.p.m. of ^3H was treated with carboxypeptidase B as described above for peptide A(i). Most of the peptide was unaffected under these conditions, though a small amount of ninhydrin-positive material, which behaved electrophoretically like arginine, was obtained. Thus the partial sequence of peptide A(ii) was Phe-(Cys-CH₂CO₂H,Y)-Arg. The remainder of the sample was subjected to the Edman degradation (Gray, 1967), and subsequently treated with diethyl pyrocarbonate followed by diazomethane. No peaks were observed in the mass spectrum of this material.

Discussion

The original experiment in the field of photo-generated reagents was done by Westheimer and his group (Singh *et al.*, 1962; Shafer *et al.*, 1966), who irradiated monodiazocetyl- α -chymotrypsin, acylated on the active-site serine-195, in the hope that the resulting carbene would label neighbouring amino acid residues at the active site. A large proportion of the label was lost by reaction with solvent, some products arose from chemical (as distinct from photochemical) reaction with neighbouring groups, and a significant amount of the first-formed α -oxo carbene suffered a Wolff-type rearrangement resulting in an alkoxyketene that was hydrolysed to *O*-carboxymethylserine. Vaughan & Westheimer (1969) subsequently found small amounts (1–3%) of product from the irradiation of the diazomalonyl half-ester of trypsin that must have arisen from insertion of the first-formed carbene into a C–H bond of an alanine residue.

Converse & Richards (1969) have used this approach with an anti-Dnp antibody. Irradiation of the complex between anti-Dnp antibody and (e.g.) *N*-Dnp-glycine diazoketone labelled up to 50% of the antibody binding sites. The loci of attachment have not yet been further delineated, but Converse & Richards (1969) state that only about half of the bound label comes from the rearranged and less reactive ketene.

The antigenic determinant

There are three criteria for a satisfactory antigenic determinant for use in this work. First, to avoid the disadvantages of cross-reaction (Singer & Doolittle,

1966; Converse & Richards, 1969), it is important that the photo-precursor of the reactive nitrene should be stable under physiological conditions so that an antibody specific for the precursor itself can be isolated. Secondly, the reactive species generated by photolysis should not be susceptible to intramolecular rearrangement to a less reactive intermediate such as a ketene. Thirdly, photolysis must be achieved by irradiation at wavelengths well clear of the absorption maxima for antibody protein.

Of all diazo- and azido-compounds which are photochemical precursors of carbenes and nitrenes (Gilchrist & Rees, 1969; Lwowski, 1970), the first criterion is satisfied only by aryl azides. These azides are chemically stable, are not particularly susceptible to photochemical rearrangement, and with appropriate substituents in the ring may be photolysed to the aryl nitrenes at wavelengths above 350 nm.

The antigenic determinant chosen was the 4-azido-2-nitrophenyl group. The nitro group was included for three reasons; it facilitates the preparation of the antigen (see the Experimental section), it shifts the absorption maximum of the hapten into the visible region away from the u.v. absorption of the protein, and it probably decreases the half-life (i.e. increases the reactivity) of the nitrene (Reiser & Leyshon, 1970).

Antigen

4-Fluoro-3-nitrophenyl azide was obtained in good yield from 4-fluoro-3-nitroaniline by diazotization followed by treatment with NaN_3 ; under slightly different conditions (Hodgson & Nixon, 1931) the initial diazotization is reported to give 2-nitrobenzene 4-diazo-1-oxide. The orientation of substituents in the Nap group minimizes the likelihood of intramolecular reactions after formation of the required nitrene, and also facilitates nucleophilic displacement of fluoride by the carrier protein. For comparison, the rate of reaction of 4-fluoro-3-nitrophenyl azide with aqueous methylamine is approx. 10^{-3} times the rate of reaction of fluoro-2,4-dinitrobenzene.

The antigen was readily prepared from 4-fluoro-3-nitrophenyl azide and bovine γ -globulin, the ϵ -amino groups of lysine residues being converted into ϵ -Nap groups. In the absence of u.v. irradiation, the azide group of the antigen was unchanged over a period of weeks at 35°C and it will therefore not decompose thermally in the living rabbit, during immunization.

Photolabelling experiments

The anti-Nap antibody absorbed, noncovalently, 1.8–2.0 mol of hapten/mol of antibody. After

photolysis at 4°C, 1.1–1.3 mol of hapten was not removable by washing with trichloroacetic acid. The affinity constant of the covalently labelled antibody was too low to be measured by equilibrium dialysis. Hence it seems that the photogenerated radioactive label is localized specifically within the antibody combining site. As has been shown in a recent application of the use of aryl azides to label the acetylcholine receptor at the end-plate of mouse diaphragm (Kiefer *et al.*, 1970), the use of shorter wavelengths accelerates the photolysis considerably, probably without significant damage to the system.

Digestion of the labelled anti-Nap antibody

The labelled protein was separated into light and heavy chains (Fig. 1), the molar ratio of radioactive label in heavy and light chains being 2.5:1. This supports the view that both heavy and light chains are involved in the binding site; the predominance of heavy-chain labelling is consistent with the hypothesis that the heavy chain contributes more to the structure of the antigen-binding site than does the light chain.

Cleavage of the heavy chain at methionine residues with cyanogen bromide yielded the fragment C-1 (Fig. 2), containing 0.42 mol of [³H]lysine/mol of fragment C-1. This means that there is only 0.03 mol of label covalently attached elsewhere in the heavy chain, other than on fragment C-1. The C-1 fragment represents the *N*-terminal 240 residues of the heavy chain, which means that 95% of the label is attached to the light chain and to the *N*-terminal half of the heavy chain. This emphasizes the non-random nature of the covalent labelling.

Tryptic digestion of fragment C-1 (see Fig. 3) yielded two radioactive peaks, one of which, peak A, contained 32% of the ³H in fragment C-1, amounting to 19% of the ³H in the separated heavy chain. The radioactive peptides in peak A were separated by paper electrophoresis into two peptides A(i) and A(ii) (Fig. 4). The sequence of peptide A(i) was (X,Ala)-Arg, and of peptide A(ii), Phe-(Cys-CH₂CO₂H,Y)-Arg.

In more recent experiments, with single-allotype rabbits and only slightly modified procedures, we have been unable to achieve high covalent-labelling efficiencies. Typically, irradiation of the antibody-hapten conjugate results in 0.5–0.7 mol of covalently bound hapten/mol of antibody (Press *et al.*, 1971), and unlabelled material can be separated from labelled antibody by affinity chromatography. The ratio of heavy- to light-chain labelling is higher (3:1 to 5:1) than that reported here, though there is a significant loss of label on cyanogen bromide digestion of the heavy chains. The reasons for these

differences are not apparent, but they do not affect the conclusions of the present work.

Conclusions

Sequence information of isolated peptides is of little value alone, but it is helpful to compare the sequences of peptides A(i) and A(ii) with those of pooled rabbit IgG heavy chains (Fruchter *et al.*, 1970; Mole *et al.*, 1971). Residues 91–94 are -Phe-Cys-Ala-Arg-, and it seems probable that the label is attached to a half-cystine residue in peptide A(i) and to an alanine residue in peptide A(ii). More importantly, residues 91–94 are just next to the major hypervariable region of rabbit heavy chain, which runs from residue 95 to 105. It therefore seems that the reagent has labelled amino acids close to this hypervariable region, in agreement with the reasonable postulate that antibody specificity arises primarily from sequence differences in the hypervariable regions.

It is worth noting that had labelling only occurred within the hypervariable region, we might well have failed to find it. Presumably there is a large number of slightly different sequences from residues 95–105, and the problems of peptide separation could well preclude the isolation of adequate quantities of material of unique sequence from this region. It is possible that much of the radioactivity associated with the longer peptides in peak B (Fig. 3) arises from labelling in the hypervariable region. Peptides with arginine-94 labelled would also be in peak B, since tryptic cleavage would not occur at such a heavily modified arginine residue. So the success of the approach may in this case depend on the relatively long life-times of aryl nitrenes, which are as long as 10⁻⁴ s for simple aryl nitrenes in a soft polystyrene matrix (Reiser *et al.*, 1968). Long reagent life-times would give the nitrene time to react with nearby parts of the antibody binding site. This interpretation must remain a postulate, until a homogeneous protein with Nap-binding capacity is studied.

Studies by the more conventional approach of affinity labelling with diazonium salts provide some support for the present conclusions. Thorpe & Singer (1969) tentatively concluded that a labelled dipeptide Thr-Tyr, isolated from the heavy-chain of rabbit anti-Dnp antibodies, was close to the major hypervariable region, at positions 94 and 95. Franěk (1971) has isolated labelled tyrosine residues at positions 33 and 93 in the light chains from pig anti-Dnp antibody, each of these residues being close to the major regions of hypervariability. Both of these studies used very low concentrations of labelling reagent since reagent concentrations giving higher extents of labelling also lead to a high proportion of randomly labelled protein. The present method does

not suffer from this disadvantage, and the analysis of antibody covalently labelled in more nearly stoichiometric proportions has been possible.

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References

- Baker, B. R. (1967) *Design of Active-Site-Directed Irreversible Enzyme Inhibitors*, J. Wiley and Sons, New York
- Cebra, J. J., Givol, D. & Porter, R. R. (1968) *Biochem. J.* **107**, 69
- Cohen, L. A. (1968) *Annu. Rev. Biochem.* **37**, 695
- Converse, C. A. & Richards, F. F. (1969) *Biochemistry* **8**, 4431
- Edstrom, R. (1968) *Anal. Biochem.* **26**, 304
- Eisen, H. N. (1964a) *Methods Med. Res.* **10**, 94
- Eisen, H. N. (1964b) *Methods Med. Res.* **10**, 106
- Eisen, H. N. (1967) *Methods Immunol. Immunochem.* **1**, 351
- Fleet, G. W. J., Porter, R. R. & Knowles, J. R. (1969) *Nature (London)* **224**, 511
- Forster, M. O. & Fierz, H. E. (1907) *J. Chem. Soc. London* **91**, 864
- Franěk, F. (1971) *Eur. J. Biochem.* **19**, 176
- Frangione, B. & Milstein, C. (1968) *J. Mol. Biol.* **33**, 893
- Fruchter, R. G., Jackson, S. A., Mole, L. E. & Porter, R. R. (1970) *Biochem. J.* **116**, 249
- Gilchrist, T. L. & Rees, C. W. (1969) *Carbenes, Nitrenes and Arynes*, Nelson, London
- Gray, W. R. (1967) *Methods Enzymol.* **11**, 469
- Haimovich, J., Givol, D. & Eisen, H. N. (1970) *Proc. Nat. Acad. Sci. U.S.* **67**, 1656
- Hodgson, H. H. & Nixon, J. (1931) *J. Chem. Soc. London* **134**, 2272
- Kamerling, J. P., Heerma, W. & Vliegthart, J. F. G. (1968) *Org. Mass Spectrom.* **1**, 351
- Katz, A. M., Dreyer, W. J. & Anfinsen, C. B. (1959) *J. Biol. Chem.* **234**, 2897
- Kiefer, H., Lindstrom, J., Lennox, E. S. & Singer, S. J. (1970) *Proc. Nat. Acad. Sci. U.S.* **67**, 1688
- Kinard, T. E. (1957) *Rev. Sci. Instrum.* **28**, 293
- Lwowski, W. J. (1970) *Nitrenes*, Interscience, New York
- Michl, H. (1951) *Monatsh. Chem.* **82**, 489
- Mole, L. E., Jackson, S. A., Porter, R. R. & Wilkinson, J. M. (1971) *Biochem. J.* **124**, 301
- O'Donnell, I. J., Frangione, B. & Porter, R. R. (1970) *Biochem. J.* **116**, 261
- Prahl, J. W. & Porter, R. R. (1968) *Biochem. J.* **107**, 758
- Press, E. M., Fleet, G. W. J. & Fisher, C. E. (1971) *Progr. Immunol.* **1**, 499
- Pressman, D. & Roholt, O. (1961) *Proc. Nat. Acad. Sci. U.S.* **47**, 1606
- Reiser, A. & Leyshon, L. (1970) *J. Amer. Chem. Soc.* **92**, 7487
- Reiser, A., Willets, F. W., Terry, G. C., Williams, V. & Morley, R. (1968) *Trans. Faraday Soc.* **64**, 3265
- Shafer, J., Bavanowsky, P., Laursen, R., Finn, F. & Westheimer, F. H. (1966) *J. Biol. Chem.* **241**, 421
- Singer, S. J. & Doolittle, R. F. (1966) *Science* **153**, 13
- Singh, A., Thornton, E. R. & Westheimer, F. H. (1962) *J. Biol. Chem.* **237**, PC3006
- Spackman, D. H., Stein, W. H. & Moore, S. (1958) *Anal. Chem.* **30**, 1190
- Thorpe, N. O. & Singer, S. J. (1969) *Biochemistry* **8**, 4523
- Vallee, B. L. & Riordan, J. F. (1969) *Annu. Rev. Biochem.* **38**, 733
- Vaughan, R. J. & Westheimer, F. H. (1969) *J. Amer. Chem. Soc.* **91**, 217
- Wilkinson, M. (1967) Ph.D. Thesis, University of London