The Utility of Photo-Affinity Labels as 'Mapping' Reagents

A STUDY OF SUB-POPULATIONS OF A SPECIFIC RABBIT ANTIBODY BY USING STRUCTURALLY RELATED PHOTO-AFFINITY REAGENTS

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A specific rabbit antibody against the 4-azido-2-nitrophenyl determinant was photo-labelled by the homologous hapten ϵ -(4-azido-2-nitrophenyl)-L-lysine, and by the close structural isomer ϵ -(5-azido-2-nitrophenyl)-L-lysine. The extents of covalent labelling of the antibody-binding site were assessed by using radioactive haptens and exhaustive displacement dialysis, which leaves the unlabelled sites empty but largely intact. A single photolysis of hapten-antibody complex suffices to label those sites that are capable of being labelled. Although there is considerable overlap among sub-populations of antibody that will bind the two haptens non-covalently, sites that can be covalently labelled by one reagent cannot be labelled by the other.

One of the most general chemical methods for the 'mapping' of ligand-binding sites is that of photo-affinity labelling, in which a photolysable ligand analogue is used to generate a highly reactive intermediate in the binding site of the macromolecule (Knowles, 1972). This approach has been used successfully in the antibody field, where it has proved possible to locate some of the regions of the immunoglobulin molecule that are labelled by a photolabile haptenic group, and directly to implicate the hyper-variable sequence loci in antibody specificity (Fleet *et al.*, 1969, 1972; Press *et al.*, 1971; Fisher & Press, 1974).

A fundamental question about the use of photo-generated reagents in the location of specific binding loci of macromolecules is whether, by varying the position of the photolabile group on the ligand, it is possible to do a real 'mapping' operation in which different parts of the binding site are labelled by re-

agents of only slightly different structure. To investigate this possibility, we have looked at the labelling of a specific antibody by the photolabile hapten [ϵ -(4-azido-2-nitrophenyl)-L-lysine: ϵ -Nap-lysine (I)] used to elicit the antibody originally, and by a related photolabile material [ϵ -(5-azido-2-nitrophenyl)-L-lysine: ϵ -isoNap-lysine (II)] in which the azide group has a slightly different orientation. We further hoped that some light would be thrown on the question of the binding-site structure of sub-populations of a normally elicited specific antibody preparation.

We report here the extent of labelling of anti-Nap antibody by the nitrenes derived from the two structurally related azidonitrophenyl haptens mentioned above, and the extent to which the labelling by the two materials is mutually exclusive.

Experimental

Materials

Anti-Nap antibody from allotype Aa3 rabbits, and ϵ -(4-azido-2-nitrophenyl)-L-[4,5- ^3H]lysine (sp. radioactivity 1.14×10^5 c.p.m./ μmol) were kindly given by Dr. E. M. Press, and were prepared as described previously (Fleet *et al.*, 1972).

3-Fluoro-4-nitrophenyl azide. 3-Fluoro-4-nitroaniline (3.16 g), prepared by the method of Hodgson & Nicholson (1941), was suspended in a mixture of water (45 ml) and conc. HCl (5 ml) and diazotized at 0°C by the slow addition of a solution of NaNO₂ (1.5 g) in water (10 ml). The solution was then filtered, and a solution of NaN₃ (1.3 g) in water (20 ml) added slowly at 0°C; N₂ was evolved. A yellow oil separated, which was extracted with ether (2 × 20 ml), and the extracts were dried over anhydrous MgSO₄. The dried extracts were treated with decolorizing charcoal,

filtered through a small amount of silica gel, and the solvent was removed under reduced pressure. The product was a yellow oil (2.34g; yield 74%), pure on t.l.c. [silica-gel plates eluted with light petroleum (b.p. 60–80°C)–ether (4:1, v/v)]. (Found: C, 39.9; H, 2.0; N, 29.5; F, 10.24. $C_6H_3FN_4O_2$ requires: C, 39.6; H, 1.65; N, 30.7; F, 10.44%). The 1H n.m.r. spectrum (at 100MHz) in CCl_4 solution showed peaks at 1.92 τ (irregular triplet, 1 proton, H-1), 3.03 τ (singlet, 1 proton) overlapping with 3.12 τ (irregular quartet, 1 proton, H-4 and H-6). The ^{19}F n.m.r. showed one multiplet at –114.2p.p.m. from CCl_3F . ν_{max} . 2122 cm^{-1} (N_3), λ_{max} . 301 nm (in ethanol). The mass spectrum showed major peaks at m/e 182, 154, 124, 108, 96, 81.

N-Methyl-2-nitro-5-azidoaniline. 3-Fluoro-4-nitrophenyl azide (0.182g) was dissolved in a mixture of aq. 25% (w/v) methylamine (2ml) and ethanol (3ml). After 1 h in the dark at 45°C the mixture was cooled to room temperature, water (2ml) was added and orange crystals were removed by filtration. Recrystallization of this material from ethanol gave needles, m.p. 109°C (0.149g; yield 70%). (Found: C, 42.8; H, 3.66; N, 36.45. $C_7H_7N_5O_2$ requires: C, 43.5; H, 3.63; N, 36.30%). The 1H n.m.r. spectrum in CCl_4 solution showed peaks at 1.80–1.90 τ [doublet of doublets (J 8.1Hz), 1 proton, aromatic H], 3.71 τ [doublet (J 1Hz), 1 proton, aromatic H], 6.98 τ [doublet (J 4Hz), 3 protons, *N*-methyl]. λ_{max} . 242 (ϵ 15300), 328 (ϵ 8850), 438 (ϵ 6230) nm [3% (v/v) ethanol in water]. ν_{max} . 3380 (NH), 2126 (N_3) cm^{-1} . The mass spectrum showed major peaks at m/e 193, 167, 165, 149, 119.

Photolysis of this compound occurs readily with 366nm light and can be followed by manometric detection of released N_2 or by monitoring the u.v. spectrum.

ϵ -[4,5- 3H]lysine. In a typical preparation, L-[4,5- 3H]lysine (3mCi, 12 μ mol) was freeze-dried, redissolved in water (0.5ml) and incubated with 3-fluoro-4-nitrophenyl azide (20mg), ethanol (0.5ml) and Na_2CO_3 (25mg) at 50°C in the dark for 6h. The mixture was then applied to a preparative silica-gel t.l.c. plate and eluted with butan-2-one–acetone–water (10:3:3, by vol.). In this system the most mobile component was the $\alpha\epsilon$ -di-isoNap-lysine, followed by the ϵ -isoNap- and α -isoNap-compounds. This order was established by unambiguously synthesizing ϵ -isoNap-L-lysine from *N*- α -t-Boc-L-lysine and 3-fluoro-4-nitrophenyl azide by a procedure similar to that described above and deprotecting the product with anhydrous trifluoroacetic acid at 0°C. The yields were: $\alpha\epsilon$ -di-isoNap-lysine, 10.9%; ϵ -isoNap-lysine, 11.0%; α -isoNap-lysine, 4.8%. The specific radioactivity of the desired ϵ -isoNap-lysine was 1.345×10^8 c.p.m./ μ mol.

2,4-Dinitrophenyl- ϵ -aminohexanoic acid. This was prepared as described by Carsten & Eisen (1951).

L-[4,5- 3H]Lysine was obtained from The Radiochemical Centre (Amersham, Bucks., U.K.).

Methods

U.v. spectra were measured with a Pye Unicam SP.1800 instrument. I.r. spectra were recorded with a Perkin–Elmer 237 recording spectrophotometer. Mass spectra were determined by Dr. R. Aplin in this department with an A.E.I. MS9 instrument. N.m.r. spectra (1H) were recorded by Mrs. E. Richards in this department with a Perkin–Elmer R14 instrument at 100MHz. ^{19}F n.m.r. spectra were recorded with a Perkin–Elmer R32 machine at 84.6MHz.

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

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

Microanalyses were performed by Dr. Strauss in this department.

Binding studies. The extent of non-covalent binding of radioactive hapten to the antibody was determined as follows. A fourfold molar excess of the appropriate radioactive hapten was mixed with antibody and incubated for 2h at 4°C in the dark. The mixture was then passed down a Sephadex G-25 column (20cm $\times$ 3.5cm) equilibrated with and eluted with 0.1M-Tris–HCl buffer, pH7.5, containing 0.5% NaCl (w/v) and 0.1 μ M of the appropriate radioactive hapten, at 4°C. The flow rate was approx. 2ml/min. Hapten: antibody ratios were determined by scintillation counting of the protein-containing fractions and are averaged over all such fractions. The excess of hapten is eluted several void volumes after the protein and there is no overlap of components. Protein concentration was estimated from the absorbance at 280nm by using $E_{0.1\%}^{10mm} = 1.40$. A correction for bound hapten was applied by subtracting $E_{360}^{10mm} \times 0.39$ from the absorbance at 280nm.

Photolabelling. The non-covalent complex of antibody and radioactive hapten, at the concentrations eluted from the Sephadex column (protein concentration 0.05–0.20mg/ml) was irradiated with two Mazda 125W MB/V pearl-glass lamps at 0°C in a $NaNO_2$ bath for 24h. The sample was approx. 10cm from the lamps.

Dialysis to remove non-covalently linked hapten. After irradiation, each sample was subjected to the following exhaustive dialysis treatment. All operations were performed at 4°C. First the sample was dialysed for 24h against *N*-dinitrophenyl- ϵ -aminohexanoic acid (1mM) in 25mM-Tris–HCl buffer, pH7.3, 0.5M in NaCl. Then the sample was dialysed for 24h against two changes of buffer (containing no *N*-dinitrophenylaminohexanoic acid). The above sequence was then repeated.

Separation of chains. Antibody was mildly reduced, alkylated and separated into heavy and light chains by the method of Prahl & Porter (1968). The extinction coefficients of the chains were those quoted by Crumpton & Wilkinson (1963).

Results and Discussion

The experiments were designed in two stages. In the first the extent of binding to and of photolabelling of anti-Nap antibody by each of the haptens I and II was investigated, and the distribution of label between heavy and light chains was found. In the second stage, 'cross-over' labelling was done, in which antibody labelled by compound (I) was allowed to bind and be photolabelled by compound (II), and conversely. The first experiment therefore aimed to provide information about differences in the binding capacity and covalent reaction of the antibody with compounds (I) and (II), and the second experiment was designed to see if there was mutual exclusiveness in the labelling by compounds (I) and (II).

Binding of haptens to antibody molecules is conveniently estimated by the amount of hapten that the protein retains on gel filtration. If the dissociation constant for the complex is less than about $1\mu\text{M}$, the protein will normally retain close to 1 mol of ligand/mol of binding site. A fourfold molar excess of hapten over binding sites was normally incubated at 4°C , and then subjected to gel filtration. The stoichiometry of the protein-hapten complex that was eluted from the column was measured, and the solution then subjected to irradiation at wavelengths greater than 400nm under conditions that do not significantly affect the ligand-binding capacity of the antibody.

To determine the amount of covalently attached hapten one can precipitate the protein with trichloroacetic acid and measure the amount of protein-bound radioactivity. In these experiments, however, it was important not to destroy the protein. Accordingly the complex, containing both covalently attached and non-covalently bound hapten, was subjected to exhaustive displacement dialysis. It was found that dialysis in 0.5M salt against the weakly cross-reacting material *N*-2,4-dinitrophenyl- ϵ -aminohexanoic acid resulted in the removal (to a constant value) of non-covalently bound radioactive hapten, and the displacing ligand could then easily be removed by dialysis. A repeat of the displacement dialysis caused little or no further decrease in bound radioactivity. Further, the successful removal of the displacing ligand was checked spectrophotometrically. In this way, labelled antibody was obtained, the radioactivity of which was due only to covalently attached hapten. (This was checked on portions of labelled antibody, by the trichloroacetic acid precipitation method.)

The extent of labelling of a macromolecule M by a photogenerated labelling reagent L is a function of

Scheme 1. Pathway for photosensitive ligand (L) labelling of macromolecule (M)

L* is the reactive species and L' is the product(s) of reaction with solvent.

the lifetime of the reactive species (L*) and of the partitioning of that species between site labelling (to L-M) and diffusion out of the site leading to reaction with solvent (to L') (Scheme 1).

The reaction of the reactive intermediate with water (k_{+3}) is likely to be rapid compared with the recombination of L* with M (k_{-2}) [on the basis of the known reactivities of aryl nitrenes (Reiser *et al.*, 1968) and at low protein concentrations].

Clearly, the percentage labelling will be large if k_{+1} is much greater than k_{+2} . The parameter k_{+1} is a first-order rate constant averaged over the various types of labelling mechanism (concerted insertion, abstraction-recombination, cyclo-addition etc.). For the aryl nitrenes used in this study it may be estimated as 10^3 – 10^4s^{-1} from flash-photolysis experiments in various media (Reiser *et al.*, 1968). k_{+2} may be estimated from the association constant for the ligand and antibody by using the facts that their association is known to be approximately diffusion controlled ($k \approx 2 \times 10^8\text{M}^{-1}\cdot\text{s}^{-1}$) (Pecht *et al.*, 1972) and that the nitrene is structurally very similar to the hapten. Under these circumstances those antibody molecules with $K_{\text{dissoc.}} < 1\mu\text{M}$ will be efficiently labelled, those with $K_{\text{dissoc.}} > 100\mu\text{M}$ will remain essentially unlabelled. Molecules with intermediate association constants will be fully labelled only by repeated treatment with hapten followed by photolysis. (In addition to these dynamic factors, subpopulations of antibodies may exist with high association constants but within whose combining sites the nitrene either reacts to form a chemically labile linkage, or is unable for steric reasons to react with an amino acid residue at all and reacts instead with a water molecule.)

Scheme 2 shows that, as expected, the anti-Nap antibody retains more of the homologous hapten on gel filtration (1.76mol of ϵ -Nap-Lys) than of its isomer (0.92mol of ϵ -isoNap-Lys). On photolysis, covalent labelling is more extensive with the homologous hapten (0.37:0.09, mol/mol). Further gel-filtration experiments indicate that either the photolysis or the displacement dialysis or both results in

Scheme 2. Labelling of anti-Nap antibody with the radioactive haptens ϵ -Nap-lys and ϵ -isoNap-lys

'Binding' refers to hapten retained by the protein after gel filtration. The number of mol of hapten/mol of antibody (which contains two binding sites), non-covalently bound, appears as superscript. 'Labelling' refers to the photolabelling-displacement dialysis sequence (see the text). The number of mol of hapten/mol of antibody, covalently bound, appears as a subscript. The estimated error in the labelling values is ± 0.01 mol/mol, which is also the extent of labelling of non-specific rabbit IgG (immunoglobulin G) by ϵ -isoNap-lys (in twofold molar excess).

some loss of binding capacity (for ϵ -Nap-Lys, from 1.76 down to 1.52 mol/mol), but it is clear that only 10–20% of the occupied sites become covalently labelled on photolysis. It was important therefore to check that all the binding sites that could be labelled had in fact been labelled by a given hapten, and this was done by subjecting the once-labelled protein to another cycle of hapten-binding-photolabelling-displacement dialysis. Insignificant further amounts of either hapten became covalently attached on the second photolysis. This suggests that the photolabelling reaction is well-behaved, and that a single photolysis suffices to block those sites that are capable of being blocked.

The extent of covalent labelling of anti-Nap antibody by the homologous hapten (0.38 mol/mol) is less than that reported by Fisher & Press (1974) (0.5–0.8 mol/mol). These workers used the trichloroacetic acid precipitation method to assess the extent of labelling, and found that 40% of the label of the heavy chain from a pool purified by affinity chromato-

graphy was lost after CNBr digestion. They suggested that this 'represents a Nap-lysine derivative bound non-covalently to the antibody.' In agreement with this, and suggesting that the exhaustive displacement dialysis method used here is more effective at removing non-covalently bound material than is the acid precipitation technique, we have shown that considerably less (approx. 25%) of the radioactivity from the heavy chain of our labelled anti-Nap antibody is released as small fragments after CNBr digestion.

The above experiment agrees with the findings alluded to earlier (Press *et al.*, 1971; Fleet *et al.*, 1972) that when antibody that has been photolabelled is separated from unlabelled material on an affinity column, the unlabelled fraction (although its non-covalent binding capacity is unaffected) cannot be covalently labelled. These results suggest that only a particular sub-population of the specific antibody is capable of irreversible reaction with the photo-generated nitrene.

Scheme 3. Cross-over labelling of Nap-labelled antibody with isoNap-Lys and isoNap-labelled antibody with Nap-Lys

The use of abbreviations and of super- and sub-scripts is as in Scheme 2.

When the twice-labelled antibodies were separated into the heavy and light chains, it was found that the ratio of label in the chains depends very much on the reagent. The heavy:light-chain ratio of label was 5.0 for the homologous ϵ -Nap-Lys reagent, but only 1.7 for the isomeric ϵ -isoNap-Lys reagent (Scheme 2). It has been known for many years that both heavy and light chains contribute to the binding site of a specific antibody (Metzger *et al.*, 1964), and Givol and his group, using bromoacetyl affinity labels in which the reactive group is at different distances from the antigenic determinant, have elegantly shown that both polypeptide chains are within 0.5 nm of the determinant binding locus (Haimovitch *et al.*, 1970). From the present results, we can conclude that both chains contribute even to a single locus for an aromatic ring. Since the labelling pattern of the heavy and light chains is dependent on the hapten used, we may ask whether (a) the same sub-population of the anti-Nap antibody is being differently labelled by the two haptens, or (b) the two haptens, although able to bind to (at least some of) the same antibody molecules, actually label different sub-populations. This question is answered by the results in Scheme 3.

In Scheme 3 are presented the results of cross-labelling antibody that has already been labelled by the other hapten. The non-covalent binding capacity of the antibodies parallels that observed in Scheme 2, but more significantly, so does the photolabelling. Thus the material that has been labelled twice with each hapten has the same overall extent of covalent label (0.46 ± 0.01 mol/mol), and the individual contributions to the labelling are independent of the order of labelling. The homologous hapten ϵ -Nap-Lys labels to the extent of 0.36–0.38 mol/mol, and the iso-

meric ϵ -isoNap-Lys to 0.09–0.10 mol/mol. It appears therefore that the molecules that can be labelled by one reagent cannot be labelled by the other, and that different sub-populations from the original specific anti-Nap antibody preparation are attacked independently by the two isomeric nitrene reagents. This is not true of non-covalent binding, however. Of the 46% of sites that will bind ϵ -isoNap-Lys sufficiently strongly to form a non-covalent complex detectable by gel filtration, at least 34% will also bind ϵ -Nap-Lys, since 88% of all sites bind this reagent. The fact that no sub-population of antibody molecules exists that can be labelled by either reagent shows a somewhat surprising difference between the binding sites. The failure to label a site in which the hapten will nevertheless bind non-covalently means either that protein labelling often occurs to produce a chemically labile bond, or that the nitrene is bound in an orientation that leads to preferential reaction with solvent. Further, the discrimination of the labelling is very high, since generation of a nitrene at centres only about 0.2 nm apart (by using Nap- and isoNap-), leads to a mutually exclusive labelling pattern. This stresses the effectiveness of the photolabelling approach and suggests that, provided the conditions of tight binding and intermediate lifetime are properly met (see above), this approach can be used both for the 'mapping' of sites in homogeneous systems and for probing the basis of heterogeneity in systems that are not.

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