The Accessibility of Protein-Bound Dinitrophenyl Groups to Univalent Fragments of Anti-Dinitrophenyl Antibody

By C. GRAHAM KNIGHT* and N. MICHAEL GREEN
National Institute for Medical Research, Mill Hill, London NW7 1AA, U.K.

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A series of *N*-(*N*-dinitrophenylaminoalkyl)maleimides were synthesized with alkyl-chain lengths of two, four and six carbon atoms. When these compounds reacted with the thiol group of mercaptalbumin, the tryptophan fluorescence of the protein was quenched. This change in fluorescence was used to determine the rate of reaction of the Dnp (dinitrophenyl)-maleimides with mercaptalbumin. The second-order rate constants were similar to those observed in reactions between low-molecular-weight thiol compounds and maleimides. When *N*-(*N*-Dnp-aminoalkyl)succinimidomercaptalbumins were added to univalent fragments of anti-Dnp antibody the antibody fluorescence was quenched. Fluorescence-quenching titrations showed that the protein-bound Dnp groups were fully available to the antibody even when the alkyl chain was short. The apparent dissociation constants were significantly greater than that of the interaction between anti-Dnp antibody and the free hapten, 6-(*N*-Dnp)-aminohexanoate. The antibody fluorescence was quenched efficiently by [Dnp-Lys⁴¹]ribonuclease A, also with an increased dissociation constant. It could be concluded from the increase in dissociation constant that the Dnp group spent no more than 0.1% of its time in the dissociated state, available to antibody. The second-order rate constants for the association between the Dnp-mercaptalbumins and the antibody were determined and were similar in magnitude to those observed in other interactions between protein and anti-protein antibody.

In the present paper we describe a new technique for probing the environment of the amino acid residues in proteins that can be labelled with Dnp† groups. This method is based on measuring the ability of protein-bound Dnp groups to combine with univalent fragments of high-affinity antibody to Dnp. When this antibody was titrated with a series of bis-Dnp compounds with increasing separation between the Dnp groups, it was found that the compounds behaved bifunctionally when there was a chain of seven or more atoms between the Dnp groups (Green, 1969; Wilder *et al.*, 1975). This implies that a Dnp group coupled to a lysine residue of a protein will be inaccessible to such antibody unless at least two or three of the methylene groups are free to move outside the van der Waals' surface of the protein.

The method has been used to measure the availability to antibody of Dnp groups attached via the thiol group of bovine mercaptalbumin by comparing the efficiency with which they quench antibody fluorescence (Velick *et al.*, 1960) with that of 6-(*N*-

Dnp)aminohexan-1-oate. *N*-(*N*-Dnp-aminoalkyl)succinimidomercaptalbumins were prepared as models of proteins carrying an exposed Dnp group by labelling the surface thiol group with *N*-(*N*-Dnp-aminoalkyl)maleimides. The results are compared with those given by mono-Dnp-ribonuclease A carrying the Dnp group on the lysine-41 residue, [Dnp-Lys⁴¹]ribonuclease A. These Dnp-labelled proteins were chosen as models to assess the method before a study of the environment of the Dnp-lysine residues in *N*-Dnp-bovine serum albumin (C. G. Knight & N. M. Green, unpublished work).

Materials and Methods

Bovine serum albumin (crystalline, salt-free) was obtained from Armour Pharmaceutical Co., Eastbourne, East Sussex, U.K. Ribonuclease A (three-times-crystallized, salt-free) was supplied by Worthington Biochemical Corp., Freehold, NJ, U.S.A. 6-(*N*-Dnp)-aminohexanoic acid was synthesized by the method of Carsten & Eisen (1953). Dimethylformamide was distilled and the fraction of b.p. 150–151°C (500ml) was passed through a column (1.5 cm × 34 cm) of Zeo-Karb 225 (H⁺ form) (Permutit Co. Ltd., London W.4, U.K.) and stored over Zeo-Karb 225 (H⁺ form) and molecular sieve 4A. Other

* Present address: Tissue Physiology Department, Strangeways Research Laboratory, Cambridge CB1 4RN, U.K.

† Abbreviations: Dnp, dinitrophenyl; Tnp, trinitrophenyl.

reagents were obtained commercially and were of the highest purity available.

N-(N-Dnp-aminoalkyl)maleimides

Compounds with two, four and six methylene groups between the Dnp group and maleimide ring were synthesized from 1,2-diaminoethane, 1,4-diaminobutane and 1,6-diaminohexane respectively.

Mono-Dnp-diaminoalkanes

Diaminoalkane (0.04 mol) in 0.8M-HCl (50ml) was vigorously stirred with chloroform (100ml) at 21°C. To this mixture sodium 2,4-dinitrobenzenesulphonate (0.01 mol) in water (15ml) was added slowly. After stirring for 24h the aqueous layer was separated and extracted with chloroform (3×50ml). The combined chloroform extracts (250ml) were acidified with 2M-HCl (50ml) and the precipitated mono-Dnp-diaminoalkane hydrochloride was filtered. The chloroform layer was further extracted with 2M-HCl (3×50ml) and the combined acid extracts (200ml) were evaporated to dryness under decreased pressure. The crude mono-Dnp-diaminoalkane hydrochlorides were recrystallized from ethanol/water (7:3, v/v) or propan-2-ol. Yields, melting points and elemental analyses are given in Table 1.

N-(N-Dnp-aminoalkyl)maleamic acids

Mono-Dnp-alkyldiamine hydrochloride (8mmol) was dissolved in water (100ml). The solution was made alkaline by addition of 2M-NaOH (4ml). The free amine was extracted into chloroform (5×50ml) and the combined extracts were dried over anhydrous Na₂SO₄. Maleic anhydride (20mmol) was added and the solution stirred for 2h at 21°C. Precipitated solid was filtered and the chloroform removed under decreased pressure. The combined solids were dissolved in 0.2M-NaHCO₃ (250ml)

and *N-(N-Dnp-aminoalkyl)maleamic acid* was precipitated with 2M-HCl (25ml). The maleamic acids were crystallized from ethanol/2-methoxyethanol (1:1, v/v) and recrystallized from dimethylformamide by addition of 2 or 3 vol. of ethanol to the hot (80°C) solution. Yields, melting points and elemental analyses are given in Table 1.

N-(N-Dnp-aminoalkyl)maleimides

A mixture of *N-(N-Dnp-aminoalkyl)maleamic acid* (3mmol), acetic anhydride (9mmol) and anhydrous sodium acetate (1.5mmol) was heated with stirring for 10min at 85–95°C. After cooling, the mixture was placed in an ice bath. After repeated washing with ice-cold water the crude maleimide was crystallized from 2-methoxyethanol and recrystallized from dimethylformamide/ethanol as described above. Yields, melting points and elemental analyses are given in Table 1.

Preparation of mercaptalbumin

Bovine serum albumin (Armour, lot MH 2470) had a thiol content of 0.65 mol/mol determined by the method of Ellman (1959). The protein was assumed to have an $E_{1\text{cm}}^{1\%}$ of 6.8 at 278 nm (Tombs *et al.*, 1959) and a mol.wt. of 66 500 (Baldwin, 1957). The thiol content was increased by the method of Markus & Karush (1957). Bovine serum albumin (200mg) was dissolved in 0.2M-sodium phosphate, pH 7.4 (10ml). 2-Mercaptoethylamine hydrochloride (57mg, 0.5mmol) was added and the solution stirred at 21°C for 45min. The solution was adjusted to pH 5 by 2M-acetic acid and passed through a column (1cm×13cm) of Dowex 50 (X8; Na⁺ form) in the same buffer at pH 5. Fractions (3ml) with an E_{278} greater than 2.0 were combined. Mercaptalbumin solutions were stored at pH 5 and 4°C and discarded after 24h. Preparations with a thiol content in the

Table 1. *Synthesis of N-(N-Dnp-aminoalkyl)maleimides*

Details of the synthetic methods are given in the Materials and Methods section.

Compound	Formula	Yield (%)	M.p. (°C)	Elemental analysis					
				Calculated			Found		
				C	H	N	C	H	N
1-(<i>N</i> -Dnp)-1,2-diaminoethane hydrochloride	C ₈ H ₁₁ ClN ₄ O ₄	30	278	36.6	4.2	21.3	36.4	4.2	20.7
1-(<i>N</i> -Dnp)-1,4-diaminobutane hydrochloride	C ₁₀ H ₁₅ ClN ₄ O ₄	85	216	41.3	5.2	19.3	41.5	5.1	19.1
1-(<i>N</i> -Dnp)-1,6-diaminohexane hydrochloride	C ₁₂ H ₁₉ ClN ₄ O ₄	75	163	45.2	6.0	17.6	44.9	6.2	17.4
<i>N</i> -(<i>N</i> -Dnp-aminoethyl)maleamic acid	C ₁₂ H ₁₂ N ₄ O ₇	70	176	44.5	3.7	17.3	44.2	3.8	17.3
<i>N</i> -(<i>N</i> -Dnp-aminobutyl)maleamic acid	C ₁₄ H ₁₆ N ₄ O ₇	80	186	47.7	4.6	15.9	48.2	4.7	15.6
<i>N</i> -(<i>N</i> -Dnp-aminohexyl)maleamic acid	C ₁₆ H ₂₀ N ₄ O ₇	85	136	50.5	5.3	14.7	50.4	5.4	14.4
<i>N</i> -(<i>N</i> -Dnp-aminoethyl)maleimide	C ₁₂ H ₁₀ N ₄ O ₆	35	238	47.1	3.3	17.9	47.2	3.5	18.3
<i>N</i> -(<i>N</i> -Dnp-aminobutyl)maleimide	C ₁₄ H ₁₄ N ₄ O ₆	30	182	50.3	4.2	16.8	50.3	4.5	16.3
<i>N</i> -(<i>N</i> -Dnp-aminohexyl)maleimide	C ₁₆ H ₁₈ N ₄ O ₆	20	122	53.0	5.0	15.5	53.1	5.4	15.8

range 0.95–1.02 mol of thiol group/mol were used for reactions with Dnp-maleimides.

N-(N-Dnp-aminoalkyl)succinimidomercaptalbumins

Bovine mercaptalbumin (0.4 μ mol) in column buffer (2 ml) was adjusted to pH 7.0 with 2 M-NaOH. *N-(N-Dnp-aminoalkyl)maleimide* (0.4 μ mol) in dimethylformamide (0.1 ml) was added with stirring at 21°C. After 15 min, 2-mercaptoethylamine hydrochloride (4 μ mol) was added and the solution stirred for 15 min; then 2 M-HCl was added to bring the pH to 3.5 and the solution passed through a column (0.3 cm $\times$ 10 cm) of Dowex 50 (X8; Na⁺ form). Fractions (1 ml) containing *N-(N-Dnp-aminoalkyl)succinimidomercaptalbumin* were combined and adjusted to pH 8.0 with 5 M-NaOH. The absorption spectrum was recorded. The concentration of Dnp groups was calculated by assuming $\epsilon = 17500 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 363 nm (Eisen & Siskind, 1964). The protein concentration was calculated from the E_{278} after correction for the contribution of the Dnp group to the absorbance at this wavelength ($E_{278} = 0.36 E_{363}$).

Preparation of [Dnp-Lys⁴¹]ribonuclease A

Ribonuclease A (4.4 μ mol) in water (5 ml) was adjusted to pH 8.0 by 0.1 M-NaOH. 1-Fluoro-2,4-dinitrobenzene (4.5 μ mol) in acetone (45 μ l) was added with stirring at 23°C. The pH was maintained in the range pH 7.9–8.2 by the addition of 0.1 M-NaOH. After 3 h at 23°C the solution was left overnight at 4°C. The reaction mixture was adjusted to pH 6.47 by 4 M-sodium phosphate buffer, pH 6.47 (0.25 ml), and dinitrophenol was removed by passing the solution through a column (0.5 cm $\times$ 10 cm) of Dowex 1 (X8; Cl⁻ form). [Dnp-Lys⁴¹]ribonuclease A was isolated by chromatography on IRC-50 (Bio-Rex 70, –400 mesh; Bio-Rad Laboratories, Richmond, CA, U.S.A.) as described by Hirs *et al.* (1965).

Anti-Dnp antibody

Anti-Dnp antibody was produced by immunization of rabbits with Dnp- γ -globulin (Eisen, 1964) and purified by precipitation with Tnp-bovine serum albumin (Eisen *et al.*, 1967). Three preparations obtained at different stages of the immunization schedule were used. Their properties in fluorescence-quenching titrations with 6-(*N*-Dnp)-aminohexanoate are described below.

Isolation of antibody fragments

Univalent antibody fragments were prepared by digestion of whole antibody with papain under reducing conditions. The procedure was similar to that described by Porter (1959). Anti-Dnp antibody preparations were stored as (NH₄)₂SO₄ precipitates at –10°C. A portion of the precipitate (215 mg) was dissolved in 0.1 M-sodium phosphate buffer, pH 7.5, containing 2 mM-EDTA (disodium salt) (5 ml) and

dialysed against the same buffer (2 $\times$ 500 ml) at 4°C. The antibody solution (10 ml) was made 5 mM in *N*-acetylcysteine and incubated with papain (2 mg) for 2 h at 37°C. The digest was centrifuged (45000g for 10 min) and the supernatant incubated with iodoacetamide (7.5 mg) for 1 h at 21°C. This solution was dialysed against water (2 $\times$ 1000 ml) at 4°C and precipitated protein removed by centrifugation as above. The supernatant [10 ml, approx. 140 mg of protein, measured by E_{278} (McGuigan & Eisen, 1968)] was dialysed against 0.01 M-sodium acetate buffer, pH 5.4 (1000 ml), at 4°C, and the dialysed solution applied to a column (3 cm $\times$ 16 cm) of Whatman CM-52 cellulose. The column was eluted at 4°C with a linear gradient from 0.01 M-sodium acetate, pH 5.4 (800 ml), to 1.0 M-sodium acetate, pH 5.4 (800 ml), at a flow rate of 42 ml/h. Fractions (7 ml) were collected and their E_{280} values measured. A portion of the eluate was taken at 50 ml intervals and the maximum degree of fluorescence quenching by 6-(*N*-Dnp)-aminohexanoate was determined. The chromatographic pattern showed three peaks of u.v.-absorbing material. The tryptophan fluorescence of the proteins in the second and third peaks showed more than 80% quenching when their ligand-binding sites were saturated by 6-(*N*-Dnp)-aminohexanoate. The fractions corresponding to these peaks were combined separately and concentrated by ultrafiltration. To each pool (5 ml) was added (NH₄)₂SO₄ (2 g). The precipitated protein was recovered by centrifugation (10000g for 25 min) and stored at –10°C.

Fluorescence-quenching titrations

The combination of the antibody-fragment preparations with 6-(*N*-Dnp)-aminohexanoate and with Dnp derivatives of mercaptalbumin and ribonuclease A was followed by measuring the decrease in the tryptophan fluorescence of the antibody on adding the Dnp compound (Velick *et al.*, 1960). The titrations were performed at 25°C in 50 mM-sodium phosphate buffer, pH 8.0, at an antibody concentration of 0.1–0.3 μ M (with respect to binding sites). The concentration of Dnp derivative was 20–50 μ M in Dnp groups. The apparatus has been described by Green (1967). The data were corrected for internal quenching effects.

Since the value of the dissociation constant of the predominant antibody was more than one order of magnitude below that of the antibody concentration, the initial part of the titration curve was linear. The equivalence point for the titration could be determined from the intersection point of the extrapolated initial portion with the final base-line fluorescence at antigen excess. In systems where most of the protein-bound Dnp groups were available to antibody, there was no problem in determining the complete titration curve, and an

apparent dissociation constant could be calculated by the method of Green & Work (1953) from the fraction of the total antibody remaining uncombined at the equivalence point. This fraction, y , is given by (Eisen & McGuigan, 1971):

$$y = \frac{Q_{\max} - Q_0}{Q_{\max}} \quad (1)$$

where $Q_{\max}$ is the maximum observed degree of quenching in the presence of excess of antigen, and Q_0 is the degree of quenching observed at the equivalence point. The apparent dissociation constant, K_{app} , is given by

$$K_{app} = [Ab]_0 \frac{y^2}{(1-y)} \quad (2)$$

where $[Ab]_0$ is the total antibody concentration.

The three preparations of univalent antibody fragments used in these experiments each had a $Q_{\max}$ of 0.85. When the concentrations of antibody determined spectrophotometrically (McGuigan & Eisen, 1968) were compared with the concentrations determined by fluorescence-quenching titrations, each preparation was found to have 0.68 mol of Dnp-binding sites/mol. The low activity was a consequence of firmly bound dinitrophenol, often present in preparations of high-affinity anti-Dnp antibody (Stevenson *et al.*, 1970).

Reaction kinetics of mercaptalbumin with N-(N-Dnp-aminoalkyl)maleimides

Portions (5 μ l) of a solution of *N*-(*N*-Dnp-aminoalkyl)maleimide (approx. 0.2 mM, in dimethylformamide) were added to bovine mercaptalbumin (2.2 μ M) in 0.2 M-sodium phosphate buffer, pH 8.0 (2 ml). During the initial part of the titration the protein fluorescence decreased to a constant value within 4 or 5 min before the next portion of maleimide was added. After 20 μ l had been added in this manner, additions were made more rapidly to establish the final linear portion of the titration curve ($Q_{\max}$). The initial and final linear portions of the titration curve were extrapolated to give the equivalence point. Kinetic runs were made at 25°C by mixing equivalent concentrations of mercaptalbumin and *N*-(*N*-Dnp-aminoalkyl)maleimide and recording the fluorescence until no further change occurred. If $[A]_0$ is the initial concentration of reactants, the second-order rate constant, k_2 , may be calculated from the relation

$$\frac{(F_0 - F)}{(F - F_\infty)} = k_2 \cdot [A]_0 t \quad (3)$$

where F_0 is the initial fluorescence, F is the fluorescence at time t and F_∞ is the final fluorescence.

Kinetics of Dnp-protein-antibody complex-formation

Antibody solution (0.25 μ M) in 0.05 M-sodium phosphate buffer, pH 8.0 (2 ml), at 25°C was mixed with an equivalent volume of Dnp-protein solution (approx. 25 μ l), determined by previous titration. The corrected decrease in fluorescence was recorded until equilibrium was reached. When the rate of dissociation of the complex is much lower than the rate of association, as occurs when anti-Dnp antibody combines with Dnp haptens (Froese, 1968), the initial-rate data may be plotted according to eqn. (3) to give the association rate constant, k_2 .

Results

Reaction of mercaptalbumin with N-(N-Dnp-aminoalkyl)maleimides

Bovine mercaptalbumin was chosen as the Dnp-group carrier protein because the thiol group is close to the surface of the protein (Hughes, 1950; Noel & Hunter, 1972) and *N*-(*N*-Dnp-aminoalkyl)succinimidomercaptalbumins should be suitable models for proteins with freely available haptenic groups. In common with most commercial preparations, the bovine serum albumin used in this work had a thiol content of 0.65 mol of SH group/mol. This was increased to 1.0 by reduction with 2-mercaptoethylamine (Markus & Karush, 1957).

When mercaptalbumin was titrated with *N*-(*N*-Dnp-aminoalkyl)maleimides the corrected protein fluorescence was progressively quenched (Fig. 1). *N*-(*N*-Dnp-aminobutyl)- and *N*-(*N*-Dnp-amino-hexyl)-maleimide gave similar titration curves. In each case complete reaction led to 50–60% quenching of the protein fluorescence. The first portion of Dnp-maleimide quenched approx. 10% of the protein fluorescence. About 3% quenching occurred rapidly and the remainder more slowly over 4 or 5 min, suggesting that some proportion of the reagent was rapidly and reversibly bound before forming a covalent bond. When mercaptalbumin was titrated with *N*-(*N*-Dnp-aminoethyl)succinimido-2-mercaptoethanol (Fig. 1) the reversible association gave about 25% of the quenching produced by the covalently bound Dnp-maleimide at the same concentration.

When equal concentrations (2.2 μ M) of mercaptalbumin and *N*-(*N*-Dnp-aminoalkyl)maleimides were mixed at pH 8.0 and 25°C, the protein fluorescence decreased rapidly and maximum quenching was attained in about 900 s. The data were fitted to the equation for a second-order reaction (eqn. 3) and showed no significant deviation from linearity (Fig. 2). The values of the second-order rate constants for the reaction of the thiol group of mercaptalbumin with *N*-(*N*-Dnp-aminoalkyl)maleimides were $0.8 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ for the ethyl derivative $1.0 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ for the butyl derivative and

Fig. 1. Quenching of fluorescence of bovine mercaptalbumin by *N*-(*N*-Dnp-aminoethyl)maleimide

●, Dnp-maleimide (0.13 mM in dimethylformamide) was added to bovine mercaptalbumin (2.2 μM, 2 ml) in 0.20 M-sodium phosphate buffer, pH 8.0, at 25°C, as described in the Materials and Methods section; ■, fluorescence quenching produced by the same concentration of *N*-(*N*-Dnp-aminoethyl)succinimido-2-mercaptoethanol.

Fig. 2. Plot of experimental data, according to eqn. (3), for the reaction between bovine mercaptalbumin and *N*-(*N*-Dnp-aminoethyl)maleimide

Bovine mercaptalbumin (2.2 μM, 2 ml) in 0.20 M-sodium phosphate buffer, pH 8.0, at 25°C, was mixed with an equivalent concentration of Dnp-maleimide. F_0 is the initial fluorescence of the protein solution, F is the fluorescence at some time-interval after mixing and F_∞ is the final fluorescence.

$1.2 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ for the hexyl derivative. The second-order plots confirmed the occurrence of some reversible binding, since they did not pass through the origin. If the small amount of non-covalently bound Dnp-maleimide that is present is sufficiently

close to the thiol group to react intramolecularly with it, then k_2 should be represented by the sum of the two terms:

$$k_2 = k_2' + \frac{k_1}{K + [X]}$$

where k_2' is the true second-order rate constant, k_1 is the first-order rate constant for the intramolecular reaction, K is the dissociation constant of the non-covalent complex and $[X]$ is the concentration of unreacted maleimide. The reaction will still remain second-order provided that $[X] \ll K$. The value of K estimated from the degree of quenching of mercaptalbumin by *N*-(*N*-Dnp-aminoethyl)succinimido-2-mercaptoethanol (Fig. 1) is about $2 \times 10^{-5} \text{ M}$, so this condition was fulfilled in these experiments.

Preparation and properties of *N*-(*N*-Dnp-aminoalkyl)-succinimidomercaptalbumins

Preparation of these derivatives by reaction of equivalent molar concentrations of mercaptalbumin and Dnp-maleimide at pH 7.0 gave products with degrees of substitution in the range 0.70–0.95 mol of Dnp groups/mol. In general, the longer the alkyl chain, the higher the degree of substitution. The lower degrees of substitution were surprising, since the half-time of the reaction under the conditions of reaction is approx. 0.5 s. The *N*-(*N*-Dnp-aminoalkyl)-maleimide and mercaptalbumin solutions were freshly prepared. Hydrolysis of the maleimide is almost certainly negligible, since *N*-ethylmaleimide has a half-life of 36 h at pH 7.0 and 20°C (Heitz *et al.*, 1968). At concentrations of Dnp-maleimide greater

than equivalence, more than 1 mol of Dnp group/mol was bound, as might be expected, since maleimides react slowly with other nucleophilic amino acid residues (Smyth *et al.*, 1964).

The apparently low degrees of substitution obtained by using equivalent concentrations may have been caused by the use of too high a value for the extinction coefficient of the Dnp group, since it is known that this can depend markedly on the environment (Eisen & Siskind, 1964; Ettinger & Hirs, 1968). Carsten & Eisen (1953) observed that 2,4-dinitrobenzene derivatives undergo spectral shifts to shorter wavelengths on binding to bovine serum albumin. Many compounds also showed a hypochromic effect. The absorption maximum of *N*-(*N*-Dnp-aminoethyl)succinimidomercaptalbumin is at 358 nm, a 5 nm shift to the blue relative to the butyl and hexyl homologues, and it is possible that the true extinction coefficient is lowered in parallel with the blue shift.

The extent of quenching of the tryptophan fluorescence of mercaptalbumin by a single Dnp group attached to the cysteine residue by reaction with *N*-(*N*-Dnp-aminoalkyl)maleimides is given in Table 2. The results have been corrected for the contribution from the protein that remained unsubstituted. The effect of alkyl-chain length on the fluorescence is small and may reflect the uncertainties in the degrees of substitution.

In titrations of anti-Dnp antibody fragments with Dnp-mercaptalbumins the residual 40–50% fluorescence leads to an accompanying change in fluorescence opposite in direction to the quenching of the antibody fluorescence. Corrections were made as described below. The correction was very small for [Dnp-Lys⁴¹]ribonuclease A, since ribonuclease A does not contain tryptophan.

Table 2. Fluorescence of *N*-(*N*-Dnp-aminoalkyl)succinimidomercaptalbumins relative to *N*-acetyltryptophan amide at the same molar concentration

Dnp-mercaptalbumins were prepared as described in the Materials and Methods section. Fluorescence was measured in 0.1M-sodium phosphate buffer, pH 8.0, at 25°C, relative to *N*-acetyltryptophan amide (AcTrpNH₂). The observed fluorescence was corrected for the proportion of the protein that remained unsubstituted. The results of two independent experiments have been averaged.

Protein	Fluorescence relative to AcTrpNH ₂	Fluorescence relative to mercaptalbumin
Mercaptalbumin	6.4	1.00
<i>N</i> -(<i>N</i> -Dnp-aminoethyl)-	2.7	0.42
<i>N</i> -(<i>N</i> -Dnp-aminobutyl)-	3.0	0.47
<i>N</i> -(<i>N</i> -Dnp-aminoethyl)-	2.4	0.38

Fig. 3. Fluorescence quenching of univalent fragments of anti-Dnp antibody by 6-(*N*-Dnp)-aminohexanoate

6-(*N*-Dnp)-aminohexanoate (0.02M) was added to univalent fragments of anti-Dnp antibody (2 ml, 5.7 μg/ml) in 50 mM-sodium phosphate buffer, pH 8.0, at 25°C. The antibody fluorescence was measured relative to *N*-acetyltryptophan amide (0.5 μM) in the same buffer.

Titration of antibody with 6-(*N*-Dnp)-aminohexanoate

The titration of the highest-affinity antibody used in these experiments with 6-(*N*-Dnp)-aminohexanoate (Fig. 3) shows an extensive linear portion followed by curvature, implying departure from stoichiometric binding in the neighbourhood of the end point. It has been observed (N. M. Green & E. J. Toms, unpublished work) that the value of K_{app} , calculated (0.1×10^{-8} M) applies only to that fraction of the antibody population which dissociates in the region of the equivalence point. The dissociation constants of the species which bind in the initial stoichiometric region were several orders of magnitude lower. Thus when this particular antibody preparation was titrated at a low protein concentration, K_{app} decreased to 0.5×10^{-10} M, which showed that most of the antibody in this preparation was of very high affinity. Nevertheless, comparison of values of K_{app} , given by different Dnp derivatives gives an approximate estimate of relative affinities, provided that the titrations are performed at the same antibody concentration.

On account of the low dissociation constant of the predominant species of antibody present, the apparent dissociation constant (reflecting the binding

of Dnp groups by lower-affinity antibody) may increase without decreasing the initial slope of the titration. This slope will decrease only when Dnp groups are so strongly hindered that the dissociation constant increases by a factor greater than 10^3 . For the purposes of the present paper we will arbitrarily define such hindered Dnp groups as unavailable, or rather, conversely, we will take the initial slope of the titration curve, relative to that with 6-(*N*-Dnp)-aminohexanoate, as a measure of the fraction of available Dnp groups in the system. This is an over-simplification in that if there was a class of groups which would give complexes with dissociation constants between approx. 10^{-7} and 10^{-5} M they would be split arbitrarily into an available subclass and a non-available subclass. We

have adopted this approach to simplify the description of the results.

Titration of antibody with N-(N-Dnp-aminoalkyl)-succinimidomercaptalbumins

When the Dnp-labelled mercaptalbumins were added to antibody the fluorescence showed the expected initial decrease (Fig. 4). The rate of quenching was lower than that found with 6-(*N*-Dnp)-aminohexanoate and it was necessary to wait up to 4 min to obtain the equilibrium reading. During the initial stages of a titration the observed fluorescence fell, but then increased again, owing to the residual fluorescence of the Dnp-protein (Fig. 4). The observed fluorescence was corrected for this added fluorescence to give the curve corresponding to quenching of the antibody. Since these corrections increased with the volume of titrant, we have used the value of $Q_{\max}$ observed in titrations with 6-(*N*-Dnp)-aminohexanoate in calculating the apparent dissociation constants.

Three series of experiments were performed by using different preparations of univalent antibody fragments. The antibody used in the first series was produced after only two doses of Dnp- γ -globulin, whereas that used in the other series came from the same rabbits which had had a third injection of antigen resulting in greatly increased concentrations of antibody in the serum. The *N*-(*N*-Dnp-aminoalkyl)succinimidomercaptalbumins were prepared freshly for each series of titrations.

As discussed above, the proportion of protein-bound Dnp group binding to the antibody was determined by comparing the corrected initial slope of the titration curve (Fig. 4) with that of a titration with 6-(*N*-Dnp)-aminohexanoate at the same antibody concentration (Fig. 3). The fraction of available Dnp groups and the apparent dissociation constants are given in Table 3.

These results show that Dnp groups attached to the thiol groups of mercaptalbumin via the aminoalkyl-succinimide chain behave almost like the free hapten with respect to quenching efficiency and so must be in exposed positions, allowing free access of the univalent fragments of anti-Dnp antibody. The apparent dissociation constants are significantly greater than those of the hapten 6-(*N*-Dnp)-aminohexanoate, but are almost unaffected by the length of the alkyl chain. The apparently greater quenching efficiency of the Dnp-aminobutyl derivative in each series may reflect a significant error in the estimation of the Dnp concentration owing to a decrease in extinction coefficient relative to that of 6-(*N*-Dnp)-aminohexanoate.

Titration of antibody with [Dnp-Lys⁴¹]ribonuclease A

When high-affinity anti-Dnp antibody was titrated with [Dnp-Lys⁴¹]ribonuclease A, much greater

Fig. 4. Fluorescence quenching of univalent fragments of anti-Dnp antibody by *N*-(*N*-Dnp-aminohexyl)succinimidomercaptalbumin

The fluorescence-quenching titration was performed under conditions identical with those described in the legend to Fig. 3. In this experiment the observed fluorescence (●) was corrected for the fluorescence of the Dnp-protein (■) to give the fluorescence of the antibody (▲).

Table 3. Availability of Dnp groups of *N*-(*N*-Dnp-aminoalkyl)succinimidomercaptalbumins to univalent fragments of anti-Dnp antibody

Availability is defined as the ratio of the initial slope of a titration with Dnp-protein to the initial slope of a titration with 6-(*N*-Dnp)-aminohexanoate at the same antibody concentration. Three independent experiments were performed by using different preparations of univalent fragments of anti-Dnp antibody as described in the text. The antibody preparations were identical with respect to $Q_{\max}$. (0.85) and the number of Dnp-binding sites/mol (0.68). The apparent dissociation constants, K_{app} , are also given.

Dnp derivative	Expt. 1		Expt. 2		Expt. 3	
	Availability	$10^8 \times K_{app}$. (M)	Availability	$10^8 \times K_{app}$. (M)	Availability	$10^8 \times K_{app}$. (M)
6-(<i>N</i> -Dnp)-aminohexanoate	1.0	1.2	1.0	0.1	1.0	0.5
<i>N</i> -(<i>N</i> -Dnp-aminoethyl)-succinimidomercaptalbumin	1.0	10.2	1.2	3.3	0.8	2.4
<i>N</i> -(<i>N</i> -Dnp-aminobutyl)-succinimidomercaptalbumin	1.1	4.4	1.2	1.1	1.2	2.1
<i>N</i> -(<i>N</i> -Dnp-aminoethyl)-succinimidomercaptalbumin	1.0	2.6	1.0	1.0	1.1	1.4

Fig. 5. Scatchard (1949) plot for the binding of [Dnp-Lys⁴¹]ribonuclease A to univalent fragments of anti-Dnp antibody

Univalent fragments of anti-Dnp antibody were titrated with [Dnp-Lys⁴¹]ribonuclease A as described in the Materials and Methods section. The concentrations of free and bound ligand were calculated from the deviations from the stoichiometric binding curve determined by titration of the antibody with 6-(*N*-Dnp)-aminohexanoate. The line drawn through the experimental points has been calculated on the basis that 25% of the Dnp groups bind to antibody with a dissociation constant (K) of 0.8×10^{-7} M and 75% bind with a dissociation constant of 5×10^{-7} M.

departure from stoichiometric titration was observed, so that reliable estimates of the free hapten present could be obtained by difference, except for the first

few points along the curve. The results in Fig. 5 are plotted by the method of Scatchard (1949) and show that a reasonable approximation to the experimental points is given by the sum of two components; 25% with $K = 0.8 \times 10^{-7}$ M and 75% with $K = 5 \times 10^{-7}$ M. It is clear from this that the Dnp group on the lysine-41 residue is available to the antibody, but that the apparent dissociation constant has increased by 50-fold if the larger K is compared with K_{app} for 6-(*N*-Dnp)-aminohexanoate (which measures the dissociation of the lowest-affinity antibodies present in the population). If the lower dissociation constant is compared with that for 6-(*N*-Dnp)-aminohexanoate measured at low protein concentration ($K = 0.5 \times 10^{-10}$ M), then the increase is by a factor of 10^4 .

Kinetics of complex-formation between antibody and Dnp-proteins

The reaction between the *N*-(*N*-Dnp-aminoalkyl)-succinimidomercaptalbumins and the univalent fragments of anti-Dnp antibody was much slower than the corresponding reaction with 6-(*N*-Dnp)-aminohexanoate. The kinetics could be followed without stopped-flow equipment, provided that equivalent amounts of reagents were used rather than an excess of antigen. Kinetic studies were therefore made of the association, since there is as yet little quantitative information available about reactions between antibody and hapten linked to protein.

When the Dnp-protein was added to an equivalent volume of antibody solution at 25°C there was a fall in corrected fluorescence (Fig. 6). The initial fast phase of the reaction lasted about 50s and the equilibrium was reached after 200–300s. In the comparable reaction with 6-(*N*-Dnp)-aminohexanoate the reaction was complete in 5s.

The data were analysed by assuming an irreversible

Fig. 6. Time-course of reaction between univalent fragments of anti-Dnp antibody and Dnp-mercaptalbumins

Equivalent concentrations ($0.27 \mu\text{M}$) of antibody and *N*-(*N*-Dnp-aminoethyl)succinimidomercaptalbumin (●) or *N*-(*N*-Dnp-aminoethyl)succinimidomercaptalbumin (■) were mixed in 50 mM-sodium phosphate buffer, pH 8.0, at 25°C . The observed fluorescence, F , was read from the recorder trace at 6 s intervals. The proportion of antibody unbound is given by the ratio $(F - F_q)/(F_0 - F_q)$, where F_0 is the fluorescence of the antibody and F_q is the fluorescence when the antibody is saturated with ligand.

Table 4. Approximate second-order rate constants for the association of univalent fragments of anti-Dnp antibody and *N*-(*N*-Dnp-aminoalkyl)succinimidomercaptalbumins

Experiments were performed under the conditions described in the legend to Fig. 6. The association showed a fast initial phase followed by a slower phase. For each phase of the association the rate of dissociation was assumed to be negligible and the data were plotted according to eqn. (3) to give approximate second-order rate constants.

Alkyl-chain length of <i>N</i> -(<i>N</i> -Dnp-aminoalkyl)- succinimidomercaptalbumin	Approx. second-order rate constants ($\text{M}^{-1} \cdot \text{s}^{-1}$)	
	Fast phase	Slow phase
$-\text{[CH}_2\text{]}_2-$	2.9×10^5	2.6×10^4
$-\text{[CH}_2\text{]}_6-$	6.4×10^5	3.4×10^4

second-order association according to eqn. (3). This treatment did not give linear plots, and it was only possible to obtain approximate rate constants for the initial and later stages of the reaction. These are given in Table 4. Attachment of the Dnp group via a longer alkyl chain resulted in significantly faster reaction in both phases of the association. Since very rapid changes in fluorescence cannot be measured accurately with our apparatus, the variations in second-order rate constant may be greater than the observed range of 10^4 – $10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$.

Discussion

Studies of the rates of reaction of *N*-alkylmaleimides with thiol groups of other proteins show that for yeast alcohol dehydrogenase (Heitz *et al.*, 1968), D-amino acid oxidase (Fonda & Anderson, 1969), rabbit muscle L- α -glycerol 3-phosphate dehydrogenase (Anderson *et al.*, 1970) and papain (Anderson & Vasini, 1970; Hall & Anderson, 1974) the rates are 100–1000 times lower than those with low-molecular-weight thiol compounds (Gorin *et al.*, 1966; Heitz *et al.*, 1968). However, the thiol groups of these proteins are relatively inaccessible, since they do not react with each other to form disulphide dimers, whereas the mercaptalbumin thiol group is relatively unrestricted by steric hindrance, since dimers are formed readily in the presence of Hg^{2+} ions (Hughes, 1950) and on treatment with oxidizing agents (Straessle, 1954; Andersson, 1966). However, the thiol group may not be completely exposed, because atmospheric oxidation to dimers requires the presence of denaturing agents (Kolthoff & Anastasi, 1958).

The high reactivity towards maleimides observed here is comparable with that observed with simple thiol compounds (Gorin *et al.*, 1966) and could be a consequence of the exposed position of the mercaptalbumin thiol group. Other factors which may have enhanced the rate of reaction are local positive

charge (Heitmann, 1968) and non-covalent binding of the maleimide in close proximity to the thiol group, allowing intramolecular reaction. Some binding does occur (Fig. 2), but there is no evidence that this occurs in the immediate neighbourhood of the thiol group. However, peptide-sequence studies (King & Spencer, 1972; Brown, 1975) have shown that the thiol group has an adjacent cluster of hydrophobic and neutral residues immediately to the *N*-terminal side, so this possibility cannot be excluded. Noel & Hunter (1972) proposed that the mercaptalbumin thiol group is reasonably close to a tryptophan residue and that both these residues are adjacent to a primary fatty acid-binding site.

Dinitrophenyl groups attached via *N*-alkylsuccinimide chains to the mercaptalbumin thiol group are freely available to univalent fragments of anti-Dnp antibody. The apparent dissociation constants for the protein-bound Dnp groups are about five times greater than that for the free hapten and confirm the relatively exposed position of the thiol group suggested by earlier results (Hughes, 1950; Noel & Hunter, 1972). A similar conclusion was reached by Hull *et al.* (1975), who studied the exposure of a nitroxide spin label attached to the mercaptalbumin thiol group by alkylmaleimides of increasing chain length. They suggest that the thiol group is either located in a crevice, about 1 nm below the protein surface, or on the surface adjacent to a binding site for hydrophobic moieties.

By contrast, the Dnp group of [Dnp-Lys⁴¹]ribonuclease A binds antibody with an apparent dissociation constant much greater than that for the free hapten, but this result is also consistent with conclusions from previous evidence. In crystalline Dnp-ribonuclease the Dnp group is folded into a cleft in the molecule, away from the active site (Allewell *et al.*, 1973), and spectral perturbation by ethylene glycol (Ettinger & Hirs, 1968) was only half of that of an unbound dinitrophenyl group. It is clear from our results that the interaction with the protein surface is a reversible one and it could be concluded from the increase in dissociation constant that the Dnp group spent no more than 0.1% of its time in the dissociated state, available to antibody. This low availability may explain discrepancies between different estimates of the antigenicity of [Dnp-Lys⁴¹]ribonuclease. Eisen *et al.* (1964) obtained moderate- to high-affinity anti-Dnp antibodies ($K = 10^{-7}$ – 10^{-8} M), whereas Brown *et al.* (1967) obtained little or no response.

The second-order rate constants for the association of univalent anti-Dnp antibody fragments and Dnp-albumins were in the range 10^4 – 10^6 M⁻¹·s⁻¹. The rates of association were between 100 and 1000 times slower than the rates measured by Froese (1968) on Dnp-hapten-antibody systems, but the rate constants were similar to those observed in protein-anti-protein

systems (Dandliker & Levison, 1967; Noble *et al.*, 1969; Sachs *et al.*, 1972). If the reaction between anti-Dnp antibody and Dnp-mercaptalbumin was diffusion controlled, the rate constant would be approximately ten times slower than the hapten-antibody interaction. That the rate is decreased further may reflect the need for the protein-bound Dnp groups to be accurately aligned with the antibody-binding site during a collision for combination to occur. The increased association rate observed with the hexyl chain (Table 4) may reflect the greater conformational freedom of the Dnp group. The effect of flexibility is clearly illustrated in the association between [Dnp-Lys²⁹]insulin and its homologous anti-Dnp antibody (Lee & Froese, 1973). In this immunogen the Dnp group is attached to a flexible portion of the polypeptide chain (Blundell *et al.*, 1972) and the association rate constant is approx. tenfold greater than the maximum value that we observe in our system.

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