MONOCLONAL IMMUNOGLOBULIN G AUGMENTS HYDROLYSIS OF AN ESTER OF THE HOMOLOGOUS HAPTEN

An esterase-like activity of the antibody-containing site?

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1. Introduction

Molecular recognition through the interaction of specific binding sites is a characteristic property of antibodies and enzymes [1]. Both types of proteins bind ligands and discriminate between closely related compounds. However, while enzymes are capable of causing cleavage of covalent bonds, it is generally held that antibodies lack this property [2]. Slobin [3] examined the effect of rabbit IgG and of the homologous antibody, on the hydrolysis of *p*-nitrophenylacetate, and found no specific enhancement in the rate of hydrolysis of the labile ester by the specific IgG. However, the studies in [4–7] indicate that specific antibody directed against the hapten may enhance the rate of hydrolysis of labile esters containing the homologous hapten. Further, we have investigated the quasi-esterase properties of monoclonal IgG directed against the well-explored haptenic group, 2,4-dinitro-phenyl (DNP), towards a fluorescent-dye conjugate of the homologous ligand with a view to elucidate the specificity, the generality and the mechanism of the hydrolytic reaction.

Here, DNP-ε-amino-caproic acid was conjugated covalently through an ester bond with the fluorescent compound 7-hydroxycoumarin to give the non-fluorescent conjugate 7-[*N*-(2,4-dinitrophenyl)-6-aminohexanoxyl]–coumarin (DNP-ε-amino-caproyl–umbelliferone conjugate; DNP–CU) (fig.1). On incubation with monoclonal anti-DNP IgG from SPE-21 in a solution buffered at pH 8, this conjugate is hydrolyzed to yield umbelliferone which fluoresces when excited at 345 nm with an emission maximum at 455 nm.

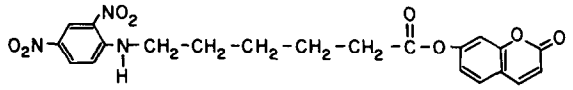

Fig.1. Structure of 7-[*N*-(2,4-dinitrophenyl)-6-amino-hexanoyl]–coumarin.

2. Materials and methods

2.1. *Reagents*

DNP-amino acids, hog liver esterase, ethoxyformic anhydride, 5,5′-dithiobis-(2-nitrobenzoic acid) were purchased from Sigma, St Louis, MO; 7-hydroxy-coumarin from Aldrich Chem., Milwaukee, WI; Dowex 1-X8 (200–400 mesh) from Dow Chem., Delaware; Sepharose–protein A from Pharmacia, Uppsala; and tetranitromethane from Fluka, Switz.

Monoclonal antibodies against DNP were obtained from hybridomas (SPE-21 and SPE-25) produced by fusion of P3-NSI/1-Ag4-1 myeloma cells with spleen cells from C57BL/6 mice immunized with DNP–keyhole limpet haemocyanin [8].

These antibodies were purified by affinity chromatography and were shown to belong to the IgG class of immunoglobulins by immunoelectrophoresis. Anti-DNP antibodies derived from rabbit and goat were obtained from Miles-Yeda, Rehovot. Rabbit

anti-DNP IgG and rabbit IgG were prepared by chromatography of whole serum on Sepharose–protein A as directed by the supplier. Anti-DNP IgG (goat) was obtained by affinity chromatography on Sepharose–DNP bovine serum albumin.

MOPC 315 and MOPC 460 IgA anti-DNP myeloma proteins were a generous gift of Dr Y. Haimowitz, Dept. of Chemical Immunology. The titer and specificity of monoclonal anti-DNP antibodies were determined by radioimmunoassay procedures, using 0.05 M Tris (pH 8) containing 0.1 M NaCl and 0.1% NaN_3 as the assay buffer. The specific antibody in 1% IgG solution of monoclonal anti-DNP antibodies determined according to [9] was 6 mg/ml. For the determination of small amounts of protein the Lowry method [10] was employed.

The assay buffer in the hydrolytic reactions was 0.05 M Tris–HCl (pH 8.0). Stock solutions of DNP-CU conjugate were prepared in chloroform and diluted to the desired concentration in assay buffer when required.

2.2. *Synthesis of 7-[N-(2,4-dinitrophenyl)-6-aminohexanoyl]-coumarin (DNP-ε-amino-caproyl–umbelliferone conjugate; DNP-CU)*

DNP-ε-amino-caproic acid was conjugated to 7-hydroxycoumarin (umbelliferone) using a carbodiimide reagent in dimethylformamide solution. The resulting DNP-CU conjugate (fig.1) was purified by thin-layer chromatography on silica gel G60 and crystallized: m.p. 124°C; ultraviolet absorption peaks at 340 nm (broad, ε 24 000) and 395 nm (ε 8000) (in ethanol).

2.3. *Fluorescence measurements*

A Farrand Ratio Fluorometer 2 was used with a constant-temperature cuvette holder and a recorder. Umbelliferone was determined in 0.05 M Tris–HCl (pH 8) using 345 nm peak wavelength for excitation and measuring emission at 455 nm. The fluorescence spectrum of umbelliferone was determined with a Perkin-Elmer MPF-44 fluorescence spectrophotometer.

3. Results

3.1. *Comparison of enzymatic and antibody-enhanced hydrolysis of DNP-ε-amino-caproyl–umbelliferone conjugate (DNP-CU)*

Incubation of DNP-CU (25 ng/tube) with hog liver esterase (0.01 U/1 ml) at 37°C in a solution buffered at pH 8 yielded a fluorescent product having the spectral characteristics of free umbelliferone with an emission maximum at 455 nm. A similar fluorescent product appeared when monoclonal anti-DNP IgG from SPE-21 (10 pmol) was substituted for the hog liver esterase. The rate of spontaneous hydrolysis of DNP-CU was minimal at pH 8.

Comparison of enzymatic hydrolysis with antibody-mediated hydrolysis of DNP-CU conjugate revealed that antibody-induced hydrolysis reaction was stoichiometric rather than catalytic. Thus, the rate of antibody-induced hydrolytic reaction of DNP-CU declined after 1 min incubation and approached zero after 6 min by which time ~2 pmol umbelliferone had been released/pmol specific antibody present in the reaction mixture (fig.2). The yield of hydrolysis was 40% under these conditions. By contrast, enzymatic hydrolysis of DNP-CU was linear for 8 min and thereafter progressed to completion within 10 min. Further addition of DNP-CU (25 ng) to the antibody-containing assay system did not result in additional release of umbelliferone, indicating satura-

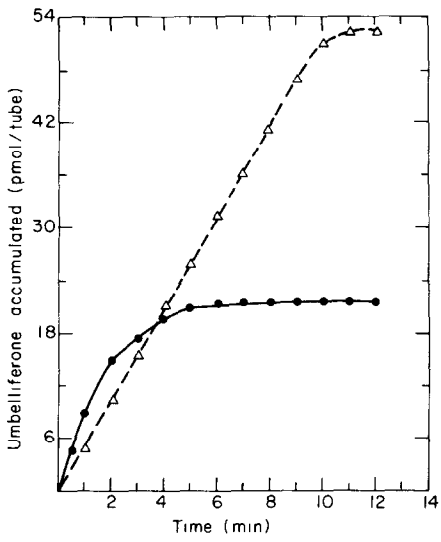

Fig.2. Enzymatic and antibody-enhanced hydrolysis of DNP-CU conjugate. Monoclonal antibody from SPE-21 (10 pmol/tube) (•——•) or hog liver esterase (0.01 U/ml) (△- - -△) was incubated with 25 ng of DNP-CU at 37°C in 1 ml Tris buffer, and fluorescence was measured continuously. The rate of evolution of fluorescence was expressed as rate of umbelliferone liberated by reference to a standard curve, after subtracting the background rate of spontaneous hydrolysis.

tion of the antibody. However, 50% of the hydrolytic activity could be regenerated by chromatography of the exhausted reaction mixture, containing IgG from SPE-21, hydrolysis products and intact substrate, on Dowex 1-X8 columns (0.5 × 1.0 cm; 200–400 mesh) equilibrated with 0.05 M NaCl–0.003 M sodium phosphate (pH 7.4). This procedure is known to cause dissociation of the DNP hapten–antibody complex [11]. This finding suggested that the slow decline in rate of the antibody-mediated hydrolysis reaction is due to failure of DNP-ε-caproic acid, formed during hydrolysis of the ester, to dissociate from the antibody combining site. By contrast, reaction products dissociate readily from the catalytic site of enzymes; thus further addition of DNP-CU (25 ng) to the enzyme-catalyzed reaction, resulted in additional release of umbelliferone.

To further test this hypothesis, the following experiment was performed. IgG from SPE-21 (10 pmol/tube) was incubated for 10 min at 37°C with an excess of the homologous hapten, DNP-ε-amino-caproic acid (200 ng), followed by addition of the ester DNP-CU (100 ng). No release of fluorescent products was observed, confirming that the free acid acted as an inhibitor. However, after Dowex chromatography as above, the hydrolytic activity of the antibody was recovered with 80% efficiency. Thus the hydrolytic properties of the antibody can be regenerated by removal of the reaction products by ion-exchange chromatography.

3.2. *The effect of various anti-DNP antibodies on the hydrolysis of DNP-ε-amino-caproyl–umbelliferone conjugate*

The ability of anti-DNP antibodies derived from different sources to cause hydrolysis of DNP-CU conjugated was compared (fig.3). MOPC 315, an IgA anti-DNP myeloma protein, was as effective as IgG from either SPE-21 or SPE-25 (data not shown), whereas MOPC 460, another IgG anti-DNP myeloma protein, and rabbit anti-DNP IgG were less effective per unit weight of protein. Normal rabbit IgG and purified goat anti-DNP antibodies had little or no effect on the rate of hydrolysis of DNP-CU conjugate. Thus the quasi-esterase activity towards the DNP esters is not a property shared by all antibodies that are able to bind this hapten.

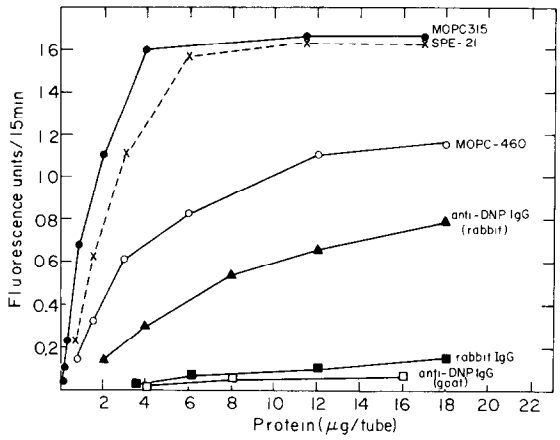

Fig.3. The effect of various anti-DNP antibodies on the hydrolysis of DNP-CU conjugate. Anti-DNP antibodies at concentrations indicated on the abcissa were incubated with 12.5 ng DNP-CU conjugate at 37°C in 0.2 ml 0.05 M Tris buffer (pH 8). The fluorescence due to liberation of umbelliferone was measured continuously for 15 min. Ordinate shows fluorescence in arbitrary units.

3.3. *Effect of various 2,4-dinitrophenyl amino acids on the antibody-enhanced hydrolysis of DNP-ε-amino-caproyl–umbelliferone conjugate*

The antibody-enhanced hydrolysis of DNP-CU conjugate was inhibited specifically by various DNP amino acids. Of the 7 derivatives tested (fig.4), DNP-ε-amino-caproic acid and DNP-γ-amino-butyric acid were the most effective competitors, whereas DNP-L-proline and DNP-L-glumatic acid had no significant effect. The same pattern of antibody specificity was observed when these DNP-amino acids were tested for their ability to compete with *N*-ε-DNP-L-[^{3}H]lysine for the binding sites of SPE-21 in a radioimmunoassay procedure (data not shown).

3.4. *Inactivation of antibody-enhanced hydrolysis of DNP-ε-amino-caproyl–umbelliferone conjugate by protein modifying agents*

In our study on the esterase-like properties of anti-steroidal antibodies we have suggested that the antibody-enhanced hydrolysis of labile esters could result from the interaction of the labile ester with a nucleophilic group present at the antibody combining site [7]. Thus, non-specific hydrolysis of steroid–umbelliferone esters could be induced by cysteine, imidazole and tyrosine [7]. Since specific reagents for modification of these groups are available [12], we examined

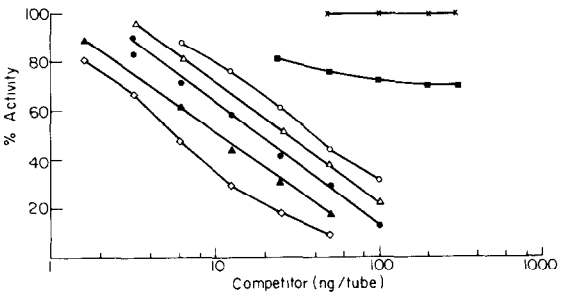

Fig.4. Effect of various 2,4-dinitrophenyl amino acids on antibody-enhanced hydrolysis of DNP-ε-amino-caproyl–umbelliferone conjugate. Monoclonal antibody from SPE-21 (5 pmol/tube) was incubated with varying concentrations of competitors in 150 μl 0.05 M Tris buffer (pH 8) for 15 min at 37°C. DNP-CU conjugate (12.5 ng/tube) in Tris buffer (50 μl) was then added to the microcuvette, and the fluorescence due to liberated umbelliferone was recorded after 10 min. The fluorescence produced by hydrolysis of DNP-CU by SPE-21 in the absence of competitor was taken as 100%. (□——□) *N*-DNP-ε-amino caproic acid; (▲——▲) *N*-DNP-γ-amino butyric acid; (●——●) *N*-ε-DNP-L-lysine; (△——△) *N*-DNP-L-ornithine; (○——○) *N*-DNP-β-alanine; (■——■) *N*-DNP-L-proline; (X——X) *N*-DNP-L-glutamic acid.

the ability of such reagents to affect the hydrolytic activity of IgG. We used 5,5′-dithiobis-(2-nitrobenzoic acid) for modifying cysteine residues [13], ethoxyformic anhydride [14] for imidazole and tetranitromethane for tyrosine residues [15]. In each case IgG from SPE-21 (25 pmol/ml) was allowed to react with 100–1000-fold excess of these reagents for 5–30 min, as specified in [13–15]. The reaction mixture was then chromatographed on Dowex 1-X 8 column to remove excess reagent. Two 1 ml fractions were collected and the concentration of protein in each fraction was determined by the Lowry method [10] to account for recovery of antibody. DNP-CU (25 ng) was then added to each fraction, and the hydrolytic activity of IgG from SPE-21 was recorded. Full hydrolytic activity of IgG from SPE-21 was retained when either ethoxyformic anhydride or 5,5′-dithiobis-(2-nitrobenzoic acid) was used. On the other hand, only 20% of the hydrolytic activity of SPE-21 was recovered when a 100-fold excess of tetranitromethane was used. This finding suggests that a nucleophilic group such as tyrosine is present at or near the antibody combining site and interacts with the DNP-CU to induce hydrolysis; and that upon nitration of the accessible tyrosine residues, the hydrolytic activity of IgG from SPE-21 is reduced or abolished.

4. Discussion

4.1. *Possible mechanism of antibody-induced hydrolysis of labile esters*

Incubation of a non-fluorescent DNP–umbelliferone conjugate with monoclonal anti-DNP IgG resulted in the generation of fluorescence. The binding of a fluorescent dye to protein often causes a bathochromic shift in the emission spectrum [11]. Since the product released by antibody from DNP-CU conjugate showed no change in the fluorescence maximum by comparison with pure umbelliferone, it seems unlikely that the resultant fluorescence is due to formation of a stable complex between DNP-CU and SPE-21. Furthermore, earlier work in our laboratory on the action of specific antibodies on steroid esters [7] has eliminated this possibility. We believe, therefore, that free umbelliferone and antibody-bound DNP-ε-amino-caproic acid were produced by antibody-induced hydrolysis of DNP-CU. The hydrolytic reaction was stoichiometric rather than catalytic. This behaviour is attributed to DNP-ε-amino-caproic acid formed during the reaction being bound to the antibody, thus causing a decline in reaction rate approaching zero as saturation of antibody is reached. Indeed, removal of DNP-ε-amino-caproic acid from the reaction mixture by ion-exchange chromatography under conditions that cause dissociation of the IgG–hapten complex, regenerated the hydrolytic activity of IgG from SPE-21.

It seems probable that the antibody-enhanced hydrolysis of DNP-CU results from interaction of the labile ester with a nucleophilic group, such as tyrosine or imidazole, present at the antibody combining site. We have shown that non-specific hydrolysis of steroid–umbelliferone esters could be induced by nucleophilic agents such as cysteine, imidazole or tyrosine [7]. The inactivation of IgG from SPE-21 by treatment with tetranitromethane, which should result in nitration of accessible tyrosine residues, suggests that tyrosine groups at or near the active site of antibody may take part in the hydrolytic reaction, possibly by formation of an activated intermediate complex.

It is known that DNP-acids interact at the combining site of anti-DNP antibodies [16]. Since the hydrolysis of DNP-CU by IgG from SPE-21 was specifically inhibited by various DNP-amino acids (fig.4), it can be inferred that the domain of immunoglobulin

responsible for specific hydrolytic activity is associated with the combining site of antibody.

4.2. *Differences in hydrolytic activity among the various DNP binding proteins*

The two anti-DNP secreting clones SPE-21 and SPE-25 used in this study bind DNP-ε-amino-caproic acid with equal affinity (1×10^9 l/mol) as determined by radioimmunoassay procedures, and both are able to enhance the rate of hydrolysis of DNP-CU (fig.3). Myeloma proteins MOPC-460 and MOPC-315 as well as rabbit anti-DNP IgG also show this esterase-like activity (fig.3), whereas goat anti-DNP IgG was devoid of hydrolytic activity. These results imply that hydrolytic activity is not a general property of the combining site of any DNP-binding immunoglobulin.

5. Concluding remarks

The demonstration that monoclonal antibodies against DNP are able to enhance the hydrolysis of DNP-CU imply that these antibodies have quasi-enzymatic properties akin to those we observed in antisteroidal antibodies. Apart from their fundamental interest, the present findings suggest that such antibodies may prove useful as tailored-to-measure reagents to induce specific hydrolytic reactions. The esterase-like properties of antibodies have also been utilized for the development of simplified immunoassay procedures for steroid hormones [6].

Acknowledgements

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