DIRECTION OF THE ACTION OF GLYCOSIDASES FROM *Limnaea stagnalis* ON THE CARBOHYDRATE GROUPS OF THE $(Fc)_5$ REGION OF IMMUNOGLOBULINS M

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UDC 547.963.1

The action of the combined glycosidases from *L. stagnalis* on the carbohydrate groups of two monoclonal immunoglobins M (Waldenström's disease) — IgM_{El} (I) and IgM_{Ser}(II) — has been studied by the semiquantitative evaluation of densitograms of glycopeptides after their separation by disc electrophoresis in polyacrylamide gel. The accessibility of the oligosaccharide groups (OGs) to the action of the glycosidases according to the positions of the OGs in a particular domain of the $(Fc)_5$ region of a IgM decreases for (I) in the sequence $C\mu3 \simeq C\mu4 >$ "hinge," and for (II) in the sequence "hinge" > $C\mu4 > C\mu3$. The difference in the accessibilities of the OGs (I) and (II) is apparently explained by differences in the conformations of (I) and (II). A hypothesis has been put forward concerning the possibility of the partial degradation of (II) by the accompanying proteases.

In recent years, prime attention has been devoted to the study of the static and dynamic aspects of the structures of the immunoglobulins, since the structures of these proteins and, in particular, their capacity for reversible and irreversible changes of conformation are connected extremely closely with their biological properties. Immunoglobulin M (IgM) has been least studied in this respect although it is apparently the most interesting one: In the immune response, IgM is the first to appear; it is the main participant in the initiation of the complement system: It is the oldest in the evolutionary aspect, and is the most complex in structure of all classes of immunoglobulins. In particular, IgM is distinguished by a high content of carbohydrates (~12%), which, calculated to the complete IgM molecule, form 50 oligosaccharide groups (OGs) of different structures and spatial mobilities [1, 2]. The oligosaccharide groupings in IgM are connected directly with its conformation. In the first place, they play an important role in the creation and maintenance of the native conformation of IgM [3-5]; in the second place, small changes in the conformation of IgM caused by a change in the conditions of the medium directly affect the spatial arrangement of the OGs [2, 6-8]; and, in the third place, the enzymatic splitting out of even part of the carbohydrates leads to substantial changes in the conformation of IgM [3-5, 9, 10].

On investigating the conformational changes of IgM in various ways, we came up against the necessity for the preliminary localization of these changes as the result of the enzymatic splitting out of part of the carbohydrate from the IgM. For this purpose, in the first place, it is important to know the direction of the action of glycosidases on IgM. Nevertheless, up to the present time it has been known only that the combined glycosidases from the large pond snail *Limnaea stagnalis* (GLS) split out the carbohydrates of the single OG in the Fab region most rapidly [11]. However, of the five OGs (calculated to the H chain of IgM) four are located in the Fc region [1].

The aim of the present investigation was to study the direction of the action of a GLS preparation on the OGs located in the $(Fc)_5$ region using as examples two monoclonal IgMs — IgM_{El} and IgM_{Ser}.

To solved this problem the native $(Fc)_5$ fragments were obtained from the native IgM and from IgM partially freed from carbohydrate with the aid of GLS (IgM*), and these were then reduced and carboxymethylated at the S—S bonds, after which they were cleaved at the

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Translated from Khimiya Prirodnykh Soedinenii, No. 3, pp. 371-375, May-June, 1984. Original article submitted April 6, 1983.

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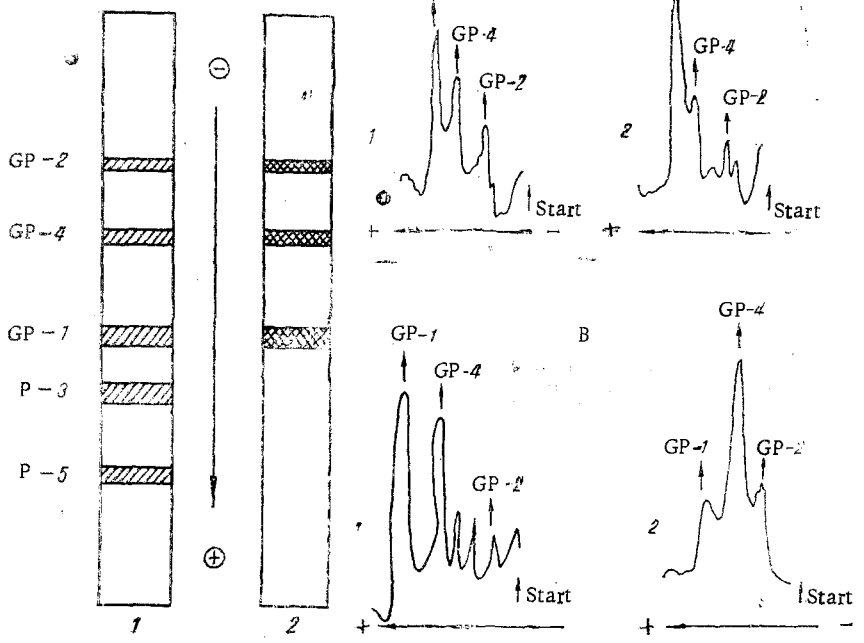

Fig. 1 Fig. 2

Fig. 1. Disc electrophoresis of the mixture of peptides and glycopeptides (MPGP) obtained on the BrCN cleavage of the $(Fc)_5$ fragments of IgM and IgM* that had been reduced and carboxymethylated at the S—S bonds: 1) staining with Coomassie G-250; 2) staining with fuchsine/sulfurous acid.

Fig. 2. Densitograms of the bands of the glycopeptides (GPs) after staining with fuchsine/sulfurous acid (see Fig. 1): A) IgM_{E1}; II) in the case of the IgM partially freed from carbohydrate (IgM*).

TABLE 1. Composition of the Mixture of Peptides and Glycopeptides (MPGPs) Formed on the Cleavage by BrCN of the Part of the H Chain of IgM Present in the Fc Fragment [12, 13]

Characteristics	Peptide (P) or glycopeptide (GP)				
	GP-1	GP-2	P-3	GP-4	P-5
Amino acid sequence [12]	326—335	337—488	490—505	507—567	569—576
Number of amino acid residues	10	152	16	61	8
Molecular weight including the carbohydrate moiety [3]	3423	20219	1758	8351	816
Hydrocarbon content, %	69,3	17,2	—	18,7	—
Position in the IgM domains [1]	"hinge"	$C_\mu 3$		$C_\mu 4$	

methionine residues by treatment with BrCN. As a result, from IgM and IgM* were obtained mixtures of peptides and glycopeptides (MPGPs) which were separated according to their molecular weights by disc electrophoresis in polyacrylamide gel in the presence of sodium dodecyl sulfate (SDS). The columns of gel were stained in pairs with Coomassie G-250 and fuchsine/sulfurous acid, identifying the individual peptides and glycopeptides (GPs), after which densitometry of the gels stained with fuchsine/sulfurous acid was carried out and the patterns obtained for IgM and IgM* were compared semiquantitatively. The starting point here was the fact that the area of the peak of a given GP on the densitogram is directly proportional to the amount of carbohydrates in this GP.

According to its primary structure, the pairs of H chain present in the Fc fragment of IgM [12, 13] should, on its cleavage with BrCN, form an MPGP of five peptides and GPs the composition of which is shown in Table 1. For IgM_{El} electrophoresis with respect to molecular weights (in the presence of SDS) did actually reveal five main bands (Fig. 1) stained by Coomassie, while three of them were also stained by fuchsine/sulfurous acid and, consequently, corresponded to the three theoretically possible GPs — GP-1, GP-2, and GP-4. The identification of the bands of the electrophoretograms was carried out in accordance with Table 1.

In the semiquantitative evaluation of the densitograms of the gels stained with fuchsine/sulfurous acid, i.e., the densitograms of the glycopeptides (Fig. 2), it was found that the ratio of the areas (S) in the case of the native IGM_{El} was $S_{GP-1}:S_{GP-4}:S_{GP-2}$ = ∿5:3:1, and for IgM^{*}_{El} this ratio was ∿8:3:1. In view of the fact that the total amount of carbohydrates split out in the preparations of IgM* was fairly large (see the Experimental part), it may be considered that there were no unaffected OGs in the IgM*. In this case, on the basis of the figures in Table 1 the following conclusions may be drawn for IgM^{*}_{El}: a) The degrees of splitting out out of the carbohydrates in GP-4 and GP-2 were the same (the ratio of the areas had not changed); b) the degree of splitting off in GP-1 was approximately 37% less than in GP-2 and in GP-4; and c) the accessibility of the OGs of IgM_{El} to the action of the glycosidases according to the position of the OGs in a particular domain decreased in the sequence $C\mu3 \approx C\mu4$ > "hinge."

The analysis of the densitograms described above in the case of IgM_{Ser} and IgM^{*}_{Ser} showed that the numbers of bands in general and of the glycopeptides in particular were greater than the theoretical number, probably as the result of a possible action of proteases accompanying the IgM_{Ser}. Nevertheless, on the basis of the results for IgM_{El} and IgM^{*}_{El} it was possible, although with difficulty, to perform an identification of the main bands. It was then found that for $IgM_{Ser}:S_{GP-1}:S_{GP-4}:S_{GP-2}$ = ∿8:5:1, and for IgM^{*}_{Ser} this ratio was ∿1:3.4:1.6. Thus, the pattern of splitting out of the carbohydrates in IgM^{*}_{Ser} was completely different from the case of IgM^{*}_{El} and this permits the following conclusion to be drawn:

a) The degree of splitting out of the carbohydrates in GP-1 was ∿82% greater than in GP-4 and ∿92% greater than in GP-2, and in GP-4, in its turn, it was ∿57% greater than in GP-2;

b) the accessibility of the OGs of IgM_{Ser} to the action of glycosidases according to the positions of the OGs in a particular domain increases in the sequence "hinge" > $C\mu4$ > $C\mu3$.

Thus, the accessibilities of the OGs to the action of glycosidases in the two IgMs studied were different, which is probably due to the different conformations of these IgMs. These differences may be either natural, formed in the course of biosynthesis, or "induced," for example through the action of the accompanying proteases on the IgM_{Ser}. The latter is extremely possible in view of the more complex composition of the MPGP from the IgM_{Ser} on electrophoresis that was mentioned above. Furthermore, under the same conditions of treatment with glycosidases, a considerably larger amount of N-acetylglycosamine was split off from the IgM_{El} (see the Experimental part). This is apparently connected with an increased breakdown of the so-called "core" of the OGs of IgM_{El}, which are present in the $C\mu3$ and $C\mu4$ domains.

EXPERIMENTAL

The preparation of immunoglobulins M (Waldenström's disease) — IgM_{El} and IgM_{Ser} — were isolated by the method described previously [14]; in the case of the Ig_{Ser} the stage of the intermediate freeze-drying of the total preparation of γ-globulins was omitted. The decarbohydrated preparations IgM^{*}_{El} and IgM^{*}_{Ser} were obtained by treating the native IgMs with the total glycosidases from *Limnaea stagnalis* [15]. The amounts of neutral carbohydrates and of N-acetylglucosamine split out were, respectively, 70 and 70-75% g for IgM^{*}_{El} and 70 and 40-45% for IgM^{*}_{Ser}.

Preparation of the Mixture of Peptides and Glycopeptides (MPGP). The $(Fc)_5$ fragments were isolated from IgM and IgM* by treatment with "hot" trypsin [16], and these were reduced and carboxymethylated at S—S bonds [17] and were then cleaved with cyanogen bromide [17]. A solution of 1 mg of the dry MPGP so obtained in 0.25 ml of 0.025 M phosphate buffer, pH

7.2, containing 0.1 M NaCl in 8 M urea was treated with 20 µl of 2% aqueous SDS solution and the mixture was then diluted to 1 ml with the initial buffer without urea.

The disc electrophoresis of the MPGP was performed in 12.5% polyacrylamide gel (3.3% cross-linkage) in 0.1 M phosphate buffer, pH 7.2, containing 0.5 M urea and 0.1% SDS (buffer A). The same buffer was used in the electrode vessels. To prepare the working gel, 12.5 g of acrylamide and 0.417 g of N,N'-methylenebisacrylamide were dissolved in 100 ml of buffer A, 1 ml of freshly-prepared aqueous ammonium persulfate solution (80 mg/ml) and 0.1 ml of N,N,N',N'-tetramethylethylenediamine were added, and the mixture was rapidly stirred, and quickly filled into the electrophoresis tubes. To avoid the cracking of the gel, the tubes had to be thin-walled, the height of the layer of the gel being 80 mm. The concentrating gel was prepared as described by Ricardo and Inmann [18]. On each tube was deposited 50-100 µl of the MPGP solution (see above), and electrophoresis was carried out for 4-6 h at a current of 8-9 mA per tube. After the end of electrophoresis, the "sausages" of gel were fixed in 12.5% trichloroacetic acid (TCA) for not less than 30 min, and stained in pairs with: a) Coomassie G-250 (a 1% aqueous solution of the dye was diluted 1:9 with 12.5% TCA and the dye was washed out with 7% acetic acid); and b) with fuchsine/sulfurous acid [19]; after the HIO_4 oxidation it was important to wash the gel well (2 days, 8 × 20 ml per "sausage"; solvent MeOH—CH_3COOH—H_2O (40:7:53)).

The densitometry of the gels that had been stained with fuchsine/sulfuric acid was carried out on an ISCO UA-5 densitometer (USA) with a rate of feed of the gel and of recording of 150 cm/h.

CONCLUSION

1. The action of the combined glycosidases from *Limnaea stagnalis* on the carbohydrate groups of the $(Fc)_5$ regions of two monoclonal immunoglobulins M (IgM_{El} and IgM_{Ser}) has been studied.

2. The accessibility of the oligosaccharide groups (OGs) to the action of glycosidases according to the positions of the OGs in a particular domain of the $(Fc)_5$ region of an IgM fell for IgM_{El} in the sequence Cµ3 ≈ Cµ4 > "hinge," and for IgM_{Ser} — "hinge" > Cµ4 > Cµ3.

3. The difference in the accessibilities of the OGs to the action of glycosidases in the IgMs studied is apparently due to differences in the conformations of these IgMs — either natural or due to the action of accompanying proteases on the IgM_{Ser}.

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ACTION OF LIGHT ON THE RELATIVE ACTIVITY OF PEROXIDASE IN THE AEROBIC OXIDATION OF LIGNIN

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UDC 547.992:577.158:541.14

It has been established that the activity of peroxidase depends to a considerable degree on the radiation dose and the spectral composition of the light. A special sensitivity of the enzyme to the action of the blue and red regions of the spectrum has been shown. In the investigation it was found that light with a wavelength greater than 520 nm promotes the liberation, and short wave light the consumption, of oxygen during the aerobic oxidation of lignin in the presence of peroxidase.

It has been shown [1] that the cambial tissue and the wood of the new annual layer of the pine contains oxidative enzymes: peroxidase and polyphenol oxidase. The substrate for these enzymes is the coniferin of the cambial tissue and its derivatives which, after the cells have died off, form lignin. Such external factors as light, temperature, soil pH, the presence of trace elements, etc., exert a great influence on the degree of lignification of the plant. According to [2], light can penetrate into wood to a depth sufficient to reach the cambial layer which, undoubtedly, has an influence on the metabolism of phenolic compounds. Ryumina [3] has shown that the formation of the bast fibers of hemp and their lignification is connected with the passage of the plant through a light-controlled stage of development. A number of workers have studied the influence of light on lignification and have obtained results showing that the processes of lignification are completed in the dark in the case of the ash [4] and in the tissues of carrot galls [5] in various young herbs and in coniferous and broad-leaved trees [6]. In a determination of the influence of light of different wavelengths, it was established that yellow-green light stimulates lignification. A number of authors have shown a maximum sensitivity of the biosynthesis of phenols to the red and blue regions of the spectrum [7-9].

We have investigated the influence of light on the catalytic activity of peroxidase and its capacity for the aerobic oxidation of lignin, which is connected with it. The activity of the enzyme is affected both by the dose and by the spectral composition of the light. According to the results obtained, with an increase in the dose of radiation there is a redistribution of the activity of the native peroxidase. Thus, while at a low dose of radiation the long-wave region of the spectrum (λ = 540-660 nm) plays the most active role, with an increase in the radiation dose the rise in the activity of the peroxidase is due to the blue region of the spectrum (λ = 365-420 nm). A considerable contribution to the activation of the enzyme is made by light with a wavelength of 545-590 nm, where, according to [10], are located the absorption bands of peroxidase (λ_{max} = 548 and 583 nm). It is obvious that under the influence of radiation in this region of the spectrum an excitation of the active center of the enzyme takes place which is reflected in its catalytic activity. The action spectrum for peroxidase is shown in Fig. 1.

A somewhat different pattern was observed when lignin is added to the reaction mixture. In this case, it was not a redistribution of the activity of the peroxidase that took place but a fall in it with an increase in the radiation dose. The maximum ratio A_λ/A_T, where A_λ is the activity of the peroxidase on irradiation at different wavelengths and A_T is the

Siberian-Research Institute of Cellulose and Cardboard, Bratsk. Translated from Khimiya Prirodnykh Soedinenii, No. 3, pp. 375-378, May-June, 1984. Original article submitted March 28, 1983.

 0009-3130/84/2003-0350$08.50