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EFFECT OF MODIFICATION ON PHYSICO-CHEMICAL AND BIOLOGICAL PROPERTIES OF HAPTOGLOBIN. REACTION WITH *N*-BROMOSUCCINIMIDE AND 2-HYDROXY-5-NITROBENZYL BROMIDE*

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N-Bromosuccinimide and 2-hydroxy-5-nitrobenzyl bromide have been used for modification of tryptophan residues in human haptoglobin (Hp) type 2-1. Modification of three exposed tryptophan residues reduced considerably both the Hp-haemoglobin interaction and binding of the antibody against the native protein. Modification of the remaining 7 - 8 tryptophan residues resulted in a complete loss of those properties. Antisera directed against Hp with the modified tryptophan residues appeared to be highly specific in immunological reactions.

Haptoglobin[1], an α_2 acid glycoprotein which forms with haemoglobin a practically irreversible complex showing catalytic activity of "true" peroxidase (Jayle, 1951), is believed to play a regulatory role in haemoglobin metabolism, and to be responsible for the non-specific reaction of organisms to neoplastic processes, infections and inflammations (Nakajima *et al.*, 1963; Putnam, 1975).

In previous papers we have examined the molecular basis of complex formation of Hp with Hb, and Hp with antibody by determining the role of the exposed lysine ε-amino groups, and tyrosine and tryptophan residues (Dobryszycka & Bec, 1971; Dobryszycka & Osada, 1973; Dobryszycka & Bec-Kątnik, 1975; Osada & Dobryszycka, 1975; Kątnik & Dobryszycka, 1977). In the present work, chemical modification of tryptophan residues was carried out with the use of two reagents, NBS and HNB, differing in the mode of action and the size of molecule (Horton & Koshland, 1965; Spande & Witkop, 1967; Barman, 1972). Application

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[1] Abbreviations used: Hp, haptoglobin; HNB, 2-hydroxy-5-nitrobenzyl bromide; NBS, *N*-bromosuccinimide; HNB-Hp, Hp with tryptophan residues alkylated by HNB; NBS-Hp, Hp with tryptophan residues oxidized by NBS; Hb, haemoglobin.

of these reagents may permit to disclose subtle differences in microenvironment of indole ring and enable estimation of the role of exposed tryptophan residues in formation of the Hp complexes with haemoglobin and antibody.

MATERIALS AND METHODS

Reagents. 2-Hydroxy-5-nitrobenzyl bromide (Sigma Chem. Co., St. Louis, Mo., U.S.A.), *N*-bromosuccinimide (Schuchardt, München, F.R.G.), Amido Black 10 B (Georg T. Gurr Ltd, London, England), Sephadex G-25 (Pharmacia, Uppsala, Sweden); other reagents were products of P.O.Ch. (Gliwice, Poland).

Human haptoglobin type 2-1 was prepared from ascitic fluid as described by Dobryszycka & Lisowska (1966). The preparations used were of 100% purity, as checked by polyacrylamide-gel electrophoresis and spectrophotometric tests (Mouray, 1966). The content of Hp was determined by the peroxidase method of Jayle (1951).

Horse haemoglobin was prepared as described by McQuarrie & Beniams (1954).

Reaction with N-bromosuccinimide. Oxidation of tryptophan residues in Hp was carried out according to Seto *et al.* (1970): to 20 mg of Hp dissolved in 2 ml of 0.2 M-acetate buffer, pH 4.1, one ml of the same buffer containing NBS at an appropriate molar excess, was added with stirring. After standing at room temperature for 30 min with occasional stirring, the mixture was applied on a column of Sephadex G-25 equilibrated with 0.1 M-acetic acid. The protein-containing fractions were pooled and freeze-dried. The number of the modified tryptophan residues in the preparation obtained, was determined spectrophotometrically and calculated according to Spande & Witkop (1967):

$$n = \frac{1.31 \times \Delta A_{278}}{5500 \times \text{molar concentration of protein}}$$

where n is the number of oxidized tryptophan residues; ΔA_{278}, the decrease in absorbance at 278 nm; 1.31, the estimated coefficient for tryptophan oxidation with NBS; 5500, the molar absorption coefficient at 278 for tryptophan oxidized with NBS.

Reaction with 2-hydroxy-5-nitrobenzyl bromide. Tryptophan residues were alkylated by the method of Horton & Koshland (1965): to 25 mg of Hp dissolved in 5 ml of 1 mM-HCl, an appropriate amount of HNB dissolved in 1 ml of acetone was added. The mixture was left at room temperature for 1 h with frequent stirring, and was fractionated on a Sephadex G-25 column. The protein-containing fractions were freeze-dried. The number of modified tryptophan residues was determined colorimetrically by measuring the absorbance at 410 nm at pH 10, taking into account the molar absorption coefficient of substituted tryptophan, 18 900 (Koshland *et al.*, 1964), and the molecular weight of Hp, 85 000 (Guinand *et al.*, 1956).

Modification of the exposed and buried tryptophan residues. To modify total tryptophan residues, Hp was treated with 8 M-urea for 1 h, then NBS was added.

The reaction with HNB, in which urea interferes, was carried out after removal of urea by dialysis.

Disc electrophoresis in 7.5% polyacrylamide gel was performed as described by Makonkaweyoon & Haque (1970) in Tris/glycine buffer, pH 8.4, at 4 mA per gel for 1 h. The gels were stained with 0.5% Amido Black in 7% acetic acid.

Immunization of rabbits. Rabbits were immunized with 0.2 mg of native or modified Hp in 0.2 ml of Freund's adjuvant, injected into the foot pad. The same dose was introduced after one week, then 2 mg portions of the antigen in physiological saline were applied intravenously at one week intervals. The antiserum against native Hp was obtained one month after immunization, whereas with the modified Hp preparations the antisera were obtained after about three months. HNB-Hp appeared to be a more potent immunogen than NBS-Hp, probably due to the content of hydroxybenzyl residue (Habeeb, 1967). After immunization, the rabbits were bled and the antisera were stored at −18°C. For immunization, the Hp preparations modified with a 100-fold and 30-fold excess of HNB and NBS, respectively (with 10.3 and 10.8 tryptophan residues modified), were used.

The double immunodiffusion tests were performed by the technique of Ouchterlony (1949) in agar gel in a wet camera for 48 h at room temperature. The plates were washed with 0.9% NaCl solution and water, dried with Whatman no. 1 filter paper, and stained for 2 min with 0.5% Amido Black in 7% acetic acid.

Quantitative precipitation tests. These were performed according to Zschocke & Bezkorovainy (1970): 100 - 200 μg of antigen dissolved in 0.1 M-Tris/HCl buffer, pH 8.0, was added to 0.5 ml of antibody solution, and incubated first for 1 h at 37°C, then for 48 h at 4°C. The samples were centrifuged, the sediments washed three times with 0.15 M-NaCl and dissolved in 1 ml of 0.1 M-NaOH. Protein was determined by the tannin micromethod (Mejbaum-Katzenellenbogen, 1955).

RESULTS

Reaction with N-bromosuccinimide. The modification of Hp was carried out using various amounts of NBS, and the characteristics of the obtained preparations is presented in Table 1. NBS applied at a 5 - 20 molar excess resulted in modification of 2.5- 9 exposed tryptophan residues. In the complex with Hb, the modified preparations showed 17 - 1% of the original peroxidase activity. In the immunodiffusion assay, they reacted with the anti-(Hp) and anti-(NBS-Hp) sera (Fig. 1A, *2*, *3* and Fig. 1C, *2*). On modification of 2.5 tryptophan residues, quantitative precipitation with the anti-(Hp) serum was lower by 50% as compared with non--modified Hp, and on modification of 5.2 tryptophan residues the precipitation was lower by 60%. With anti-(NBS-Hp) serum, the precipitation increased with the extent of modification. At 11 tryptophan residues oxidized (30-fold and higher excess of the reagent), the preparation lost the ability to activate haemoglobin and to react with the anti-(Hp) serum, but it did react with the anti-(NBS-Hp) serum (Fig. 1C, *4*, *5*). The native Hp and the NBS-Hp preparations, irrespective

of the number of modified tryptophan residues, did not react with the anti-(HNB-Hp) serum.

On modification of Hp in 8 M-urea with a 50-fold molar excess of NBS, 12 tryptophan residues were blocked and the preparation formed no complex with Hb and did not react with the antibodies.

Table 1

Characteristics of the haptoglobin preparations treated with N-bromosuccinimide

For details see Methods.

Hp preparation	NBS to Hp molar ratio	No. of modified Trp	Peroxidase activity (%)	Antigenic activity in precipitation test with serum		
				anti-(Hp) (% of native)	anti-(NBS-Hp) (% of maximum)	anti-(HNB-Hp)
Native	—	0	100.0	100.0	66.6	0
Modified	5	2.5	16.6	51.0	64.4	0
Modified	10	5.2	10.2	39.7	86.0	0
Modified	15	7.0	4.8	+	+	0
Modified	20	9.1	1.6	+	+	0
Modified	25	10.2	0	0	+	0
Modified	30	10.8	0	0	100.0	0
Modified	35	10.8	0	0	+	0
Hp in 8 M-urea	—	0	33.0	+	+	0
Hp in 8 M-urea, modified	50	11.7	0	0	0	0

+, Positive precipitation reaction.

Reaction with 2-hydroxy-5-nitrobenzyl bromide. HNB applied at a 15 - 50-fold molar excess blocked 3 - 9 exposed tryptophan residues (Table 2). The preparations showed with Hb 18 - 8% of the peroxidase activity of native Hp, 46 - 29% reactivity with the anti-(Hp) serum, and 44 - 32% reactivity with the anti-(NBS-Hp) serum. The preparation with 3 modified tryptophan residues did not react with the homologous antiserum. The reactivity of the preparations with 5 and 9 modified residues corresponded to 29 and 58%, respectively. At still higher HNB concentrations, 11 exposed tryptophan residues were modified; this resulted in a complete loss of the peroxidase activity, of the ability to react with the anti-(Hp) serum (Fig. 1A, *7*) and anti-(NBS-Hp) serum (Fig. 1C, *8*, *7*), but the preparation continued to react with the anti-(HNB-Hp) serum (Fig. 1B, *7*, *8*).

Hp treated with 8 M-urea and alkylated by a 300-fold molar excess of HNB contained 13 modified tryptophan residues and was unable to activate haemoglobin or bind antibodies.

Table 2

Characteristics of the haptoglobin preparations treated with 2-hydroxy-5-nitrobenzyl bromide

For details see Methods.

Hp preparation	HNB to Hp molar ratio	No. of modified Trp	Peroxidase activity (%)	Antigenic activity in precipitation test with serum		
				anti-(Hp) (% of native)	anti-(NBS-Hp) (% of maximum)	anti-(HNB-Hp) (% of maximum)
Native	—	0	100.0	100.0	+	0
Modified	15	2.9	18	45.6	44.0	0
Modified	30	5.2	12	+	+	29.2
Modified	50	9.1	8	29.4	32.0	58.3
Modified	100	10.3	0	0	0	100.0
Modified	150	10.8	0	0	0	+
Modified	200	11.4	0	0	0	+
Hp in 8 M-urea	—	—	33.0	+	0	0
Hp treated with 8 M-urea, modified	300	13.0	0	0	0	0

+, Positive precipitation reaction.

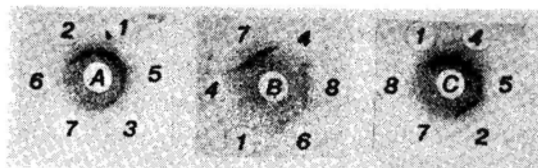

Fig. 1. Immunodiffusion patterns obtained with native and modified haptoglobin. The central well contained: in *A*, anti-(Hp); in *B*, anti-(HNB-Hp); in *C*, anti-(NBS-Hp) serum. The outer wells contained 0.05% solution of the following antigens: *1*, native Hp; *2*, NBS-Hp (2.5 Trp modified); *3*, NBS-Hp (9.1 Trp modified); *4*, NBS-Hp (10.2 Trp modified); *5*, NBS-Hp (10.8 Trp modified); *6*, HNB-Hp (9.1 Trp modified); *7*, HNB-Hp (10.3 Trp modified); *8*, HNB-Hp (11.4 Trp modified).

Polyacrylamide-gel electrophoresis of modified haptoglobin preparations. The preparations modified with a moderate molar excess of NBS or HNB showed a mobility similar to that of native Hp (Fig. 2, gels *1*, *2*, *5*, *6*). However, the second anodic fraction of NBS-Hp was separated into two bands much less distinct than in the case of HNB-Hp. When higher amounts of the reagents were used, penetration of the modified protein into the gel was lower. The cathodic fractions overlapped, and in the preparations modified at a 25- and 35-fold molar excess of NBS fraction I was not visible (Fig. 2, gels *3* and *4*).

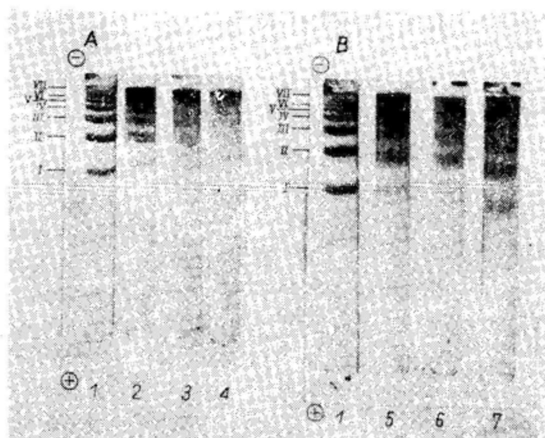

Fig. 2. Electrophoretic patterns of native haptoglobin, of Hp oxidized with NBS and Hp alkylated with HNB. Each gel was loaded with 100 μg of protein and run for 1 h at 4 mA/gel at pH 8.4. *1*, Native Hp. *A*, Haptoglobin oxidized with increasing amounts of NBS (see Table 1): *2*, NBS-Hp, 2.5 Trp modified, 16.6% of peroxidase activity; *3*, NBS-Hp, 10 Trp modified, no peroxidase activity; *4*, NBS-Hp, 11 Trp modified, no peroxidase activity. *B*, Haptoglobin alkylated with increasing amounts of HNB (see Table 2); *5*, HNB-Hp, 9 Trp modified, 8% of peroxidase activity; *6*, HNB-Hp, 10 Trp modified, no peroxidase activity; *7*, HNB-Hp, 11 Trp modified, no peroxidase activity.

DISCUSSION

Studies on the reactivity of particular amino acid residues in protein may supply valuable information on folding of the polypeptide chain (Barman, 1972; Myer, 1972). In the present work, *N*-bromosuccinimide and 2-hydroxy-5-nitrobenzyl bromide were used to modify tryptophan residues in haptoglobin, and the efficiency of modification was examined in the reaction with haemoglobin and the antibodies.

HNB (Koshland's reagent) alkylates tryptophan residues in acid medium, especially when the protein contains no sulphydryl groups (Koshland *et al.*, 1964; Horton & Koshland, 1965), as is the case with Hp. On the other hand, NBS which oxidizes the indole ring reacts also with tyrosine, methionine and cysteine residues (Spande & Witkop, 1967; Myer, 1972). However, according to Holmgren (1973), at a low concentration of NBS tryptophan residues are selectively blocked and conformation of the protein remains virtually unchanged.

It was shown previously (Dobryszycka & Bec-Kątnik, 1975; Kątnik & Dobryszycka, 1977) that tyrosine residues in Hp had an essential role in the complex formation with Hb but did not participate in the reaction with antibody. Therefore the use of different amounts of HNB, a reagent specific for tryptophan, and NBS which at higher concentration can interact also with tyrosine, enabled determination of the role of tryptophan in the two examined biological activities of haptoglobin. It should be noted that the low content of methionine in Hp permits to disregard the possible modification of methionine by NBS.

The modifying reagents were used at such concentrations that the smallest dose applied blocked about 3 tryptophan residues, because previously we have

found that modification with HNB of 3 tryptophan residues in dog Hp decreased distinctly the peroxidase activity of the Hp-Hb complex (Dobryszycka *et al.*, 1969). Now, we attempted to perform a similar modification using a reagent with different physico-chemical properties, and to examine to what extent the antigenic reactivity of the preparations obtained is correlated with the peroxidase activity.

The conversion of tryptophan residues in a Hp molecule into dihydroxyindole derivatives with the pyrrolidine ring of HNB added, or into spiroketoindole lactone formed in the reaction with NBS, distinctly affected the peroxidase activity of the Hp-Hb complex, because modification of only three out of eleven exposed tryptophan residues resulted in 80 and 85% loss of the activity.

The anti-(HNB-Hp) serum showed high specificity and reacted only with the homologous antigens, whereas the anti-(Hp) and anti-(NBS-Hp) sera formed the immunocomplexes both with native Hp and the preparations modified with moderate amounts of NBS and HNB.

On the basis of the results so far obtained on the effect of chemical modification on biological properties of haptoglobin, one can assume that haptoglobin possesses two different sites for binding Hp and antibody, which overlap in the region containing tryptophan.

The complex of Hp with antibody does not lose completely the ability to bind haemoglobin (unpublished). Hb bound with Hp blocked some antigenic determinants of Hp, 1 mole of Hb being necessary to block the maximum number of antigenic determinants in 1 mole of Hp type 1-1; however, the antisera specific for different types of Hp can discern Hp 1-1 and Hp 2-2 when these are bound to Hb (Korngold, 1965). Rogard & Waks (1977), who used the modification with NBS for studying the role of Hb tryptophan residues in complex formation with Hp, demonstrated that 2 tryptophan residues of Hb are masked by Hp after the complex formation.

The use of both reagents in higher amounts caused essential changes in the biological activities of haptoglobin; these changes are, however, hard to identify because of the possible side reactions. On polyacrylamide-gel electrophoresis, band II of the haptoglobin with only 2.5 tryptophan residues modified by NBS, was clearly resolved (Fig. 2, gel *2*) probably as a result of the peptide bond splitting. According to Spande & Witkop (1967) and Bezkorovainy *et al.* (1976) such a reaction is possible. Electrophoretic patterns of the preparations modified with various amounts of NBS and HNB point to a disturbance in the Hp structure. NBS applied at a 25 - 30-fold molar excess, causing modification of 10 - 11 tryptophan residues, destroyed completely the characteristic pattern of native Hp; this indicated disintegration of the protein and aggregation of the fragments formed.

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WPŁYW MODYFIKACJI NA WŁAŚCIWOŚCI FIZYKO-CHEMICZNE I BIOLOGICZNE HAPTOGLOBINY. REAKCJA Z *N*-BROMOBURSZTYNYLOIMIDEM I BROMKIEM 2-HYDROKSY-5-NITROBENZYLOWYM

Streszczenie

Reszty tryptofanowe w ludzkiej haptoglobinie (Hp) typu 2-1 modyfikowano przy pomocy *N*-bromoimidu kwasu bursztynowego (NBS) lub bromku 2-hydroksy-5-nitrobenzylowego (HNB). Modyfikacja trzech powierzchniowych reszt tryptofanowych w Hp znacznie redukowała zdolności tworzenia kompleksu z hemoglobiną i wiązania przeciwciał skierowanych przeciwko natywnej Hp. Modyfikacja pozostałych 7 - 8 reszt powodowała całkowitą utratę tych właściwości biologicznych. Surowice odpornościowe skierowane przeciwko Hp z powierzchniowymi resztami tryptofanowymi zablokowanych przez NBS lub HNB okazały się wysoce specyficzne w reakcjach immunologicznych.

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