STERIC ACCESSIBILITY OF TYROSINE RESIDUES IN HUMAN SERUM ALBUMIN

Ladislav MORÁVEK, Mohamed ALI SABER* and Bedřich MELOUN

Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, 166 10 Prague 6

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Human serum albumin was nitrated by an excess of tetranitromethane at pH 8·0. As shown by amino acid analysis, of the 18 tyrosine residues present in albumin about 7—7·5 residues remain unaltered, 9 residues are converted into 3-nitrotyrosine, and 1·2 residue into 3,5-dinitrotyrosine. The nitrated albumin was digested with cyanogen bromide to three fragments which comprise the whole original molecule. The individual fragments were converted into their S-sulfo derivatives and the latter digested with chymotrypsin or stepwise with trypsin and thermolysin. The yellow, nitrotyrosine-containing peptides were isolated from the digests and the positions of nitrated tyrosine residues in albumin thus located. Residues No 30, 148, 150, 161, 334, 341, 401, and 411 were identified as strongly nitrated and residues No 84, 138, 452, and 497 as medium nitrated. Residues No 140, 263, 319, 332, 353, and 367 either react weakly or were not found in nitrated form. Residue No 411 and partly also 161 were converted into 3,5-dinitrotyrosine. The accessibility of the individual tyrosine residues to the nitrating agent is discussed with respect to their positions in disulfide loops and hypothetic parts of the secondary structure of albumin.

The determination of the complete amino acid sequence of human serum albumin[1,2] (HSA) and of the bovine albumin[3] (BSA) provided a basis for rational studies on the arrangement and interactions of the molecule of this protein. Additional information on the arrangement of its 34 disulfide bonds[4] has furnished experimental evidence showing that the molecule consists of nine double loops distributed in succession along the polypeptide chain. This characteristic arrangement in which regions stabilized by disulfide bonds alternate with more flexible regions is in accordance with the varying shape of the albumin molecule observed in numerous earlier studies[5].

An earlier communication[6] was designed to contribute to the knowledge of the role which these loops play in the cooperative formation of one of the binding sites which must be arranged three-dimensionally. The obtained information on the amino acid sequence and disulfide bonds permits a two-dimensional planar scheme to be constructed, which provides partial information only on the actual sterical parameters of the molecule stabilized in formations of secondary and tertiary structure. One of the reliable chemical lines of approach to this problem represents the determination of the accessibility of tyrosine residues in various regions of the chain to tetranitromethane[7]. Human serum albumin contains 18 tyrosine residues whose positions in the chain are shown in the scheme in Fig. 1. Because of this great number of residues a study intended to isolate the nitrated peptides is a relatively difficult task. The latter can be simplified by pre-

* Visiting scientist on leave from the National Research Centre, Cairo, Arab Republic of Egypt.

iminary fragmentation[8] of nitrated albumin with intact disulfide bonds by cyanogen bromide cleavage yielding the high molecular weight fragments N, M and C (Fig. 1). The nitration of tyrosines in albumin has been studied by Malan and Edelhoch[9]. According to these authors the treatment of human serum albumin with tetranitromethane at pH 8·0 leads to nitration of approximately 50% of the tyrosine residues; the authors did not, however, identify these residues.

In this study we carried out the nitration under similar conditions, providing a possibility of comparing the results obtained, in an effort to identify the positions of tyrosine residues accessible to the nitrating reagent.

EXPERIMENTAL

Material

Human serum albumin was a product of the Institute of Sera and Vaccines, Prague. Tetranitromethane was from Spolana, Neratovice, Czechoslovakia and was redistilled before use. Chymotrypsin was prepared by the activation of chymotrypsinogen, obtained by five-fold crystallization of the commercial preparation of Léčiva, Prague. Chymotrypsin was crystallized three times with ammonium sulfate, dialyzed against 1 mM-HCl, and lyophilized. TPCK-Trypsin (trypsin treated with 1-chloro-4-phenyl-3-tosylamino-2-butanone[10]) was from Worthington Biochemical Corporation, Freehold. N. J., U.S.A. Thermolysin was a B-grade product of Calbiochem, San Diego, U.S.A. Sephadex G-25 fine, G-100, and SE-Sephadex C-25 were products of Pharmacia, Uppsala, Sweden. D,L-Tyrosine was from Nutritional Biochemicals Corporation, U.S.A. The samples of 3-nitrotyrosine were prepared by the late Dr V. Holeyšovský. The remaining chemicals used were commercial products of analytical purity.

Methods

The isolation of peptides by paper techniques was carried out on Whatman No 3 paper by chromatography in the system 1-butanol–pyridine–acetic acid–water (15 : 10 : 3 : 12 v/v), in certain cases by repeated development[11], and by two electrophoretic techniques. The separation at pH 5·6

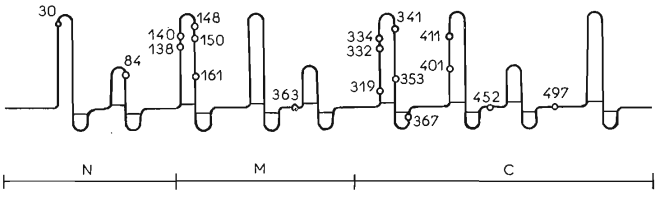


FIG. 1

Distribution of Tyrosine Residues in Molecule of Human Serum Albumin

The bars designate the individual cyanogen bromide fragments of albumin (with intact disulfide bonds): N (residues No 1—123), M (residues No 124—298), and C (residues No 299 to 585). Horizontal links show disulfide bonds.

in the system pyridine–acetic acid–water (5 : 1 : 494) was effected at 1 500 V (30 V/cm) in the descending arrangement[12]. The separation at pH 1·9 in the system formic acid–acetic acid–water (50 : 150 : 800, v/v) at 4000 V (70 V/cm) was carried out in the horizontal arrangement in the apparatus of Prusik and coworkers[13,14]. Peptide maps were prepared by a combination of electrophoresis at pH 5·6 (first direction) and by chromatography in the above system (second direction). The yellow spots of nitrotyrosine peptides were inspected after exposure of the dry chromatogram to ammonia vapors; the reference samples of an amino acid mixture and in certain cases also peptides not containing nitrotyrosine were detected by ninhydrin. Amino acid analyses were made on 20-h hydrolysates (at 110°C) of the protein material or peptides by the method of Spackman and coworkers[15] in Model 6020 Amino Acid Analyzer manufactured by the Instrument Development Workshops of the Czechoslovak Academy of Sciences, Prague. The products of tyrosine nitration were determined as described by Sokolovsky, Riordan, and Vallee[7] in the amino acid analyzer. On the basis of informative experiments carried out with standard amino acid samples, the analytical values of all tyrosine forms were corrected by +5%. Colorimetric analysis was employed especially for a rapid determination of the relative amounts of nitrated tyrosines in fractions and pure peptides. To estimate the content of 3-nitrotyrosine at 428 nm a molar extinction coeficient of 4100 (ref.[7]) was used. The molar extinction coefficient for 3,5-dinitrotyrosine at 444 nm was 6400 and was determined by measurement of a solution of this modified amino acid.

Preparation of nitrated albumin. The nitration of albumin (10 g) was carried out essentially according to Malan and Edelhoch[9]. Nitrated albumin was separated from low molecular weight products by gel filtration on a column of Sephadex G-25 (95 × 9·5 cm) equilibrated with 10 mM--NH_4HCO_3. Lyophilization afforded 9·5 g of the dry preparation of nitrated albumin.

Cleavage of nitrated albumin by cyanogen bromide and isolation of the fragments. The cleavage of albumin and the separation of the fragments were carried out as described for the fragmentation of native albumin[8]. Nitrated albumin (9·5 g) was dissolved in 230 ml of 80% formic acid and the solution was treated with 10 g of cyanogen bromide. The digestion was allowed to proceed 48 h at 5°C. The reaction mixture was freed of cyanogen bromide by gel filtration on a column of Sephadex G-25 (125 × 5·5 cm) equilibrated with formate buffer[16] (2 ml of 26% NH_4OH, 8 ml of 99% formic acid and 990 ml of water, pH 2·9). Protein-containing fractions were pooled and concentrated by ultrafiltration on Amicon UM10 membrane to a volume of 400 ml. The cyanogen bromide digest was fractionated on a column of Sephadex G-100 equilibrated by the above formate buffer (Fig. 2). Fractions designated A, C, and N + M were obtained. Fraction A contained aggregated material, which was not treated further. Fraction C, containing cyanogen bromide fragment "C", was concentrated to 250 ml by ultrafiltration. This concentrated solution was subjected to gel filtration on a column of Sephadex G-25 (47 × 6 cm), equilibrated with 0·5% acetic acid, which was also used for elution. After lyophilization the protein-containing fraction afforded 2·6 g of fragment C. The separation of fraction N + M containing cyanogen bromide fragments "N" and "M" was effected as described earlier[8]. Fraction N + M was concentrated to 100 ml by ultrafiltration. The solution was diluted with the same volume of water and applied to a column of SE-Sephadex C-25 (60 × 2·5 cm) equilibrated with 50 mM-CH_3 COONa, pH 5·0. The column with the sample was eluted stepwise by 600 ml of the above buffer and subsequently by 400 ml of 50 mM-CH_3COONa containing 8M urea. The separation was effected by elution by a linear concentration gradient of sodium chloride in the same buffer (Fig. 3). Fractions containing the separated fragments N and M were concentrated by ultrafiltration, acidified by formic acid, and 90-ml portions of the solutions were subjected to gel filtration on a column of Sephadex G-25 (57 × 6 cm) in 0·5% CH_3COOH. Protein-containing fractions were lyophilized. The final yield was 570 mg of fragment N and 1090 mg of fragment M.

Under the conditions of elution described a part of the yellow material remained adsorbed to the SE-Sephadex column.

Preparation of S-sulfo derivatives of cyanogen bromide fragments of nitrated albumin. The S-sulfo derivatives were prepared by the procedure of Pechère and coworkers[17], slightly modified in our previous study[18].

Preparation od enzymic digests of S-sulfonated fragments. For the preparation of the chymotryptic digest of fragment N and M the corresponding fraction of the effluent obtained by gel filtration of the reaction mixture after S-sulfonation was used. The solution in 0·2M-$(NH_4)_2CO_3$,

TABLE I

Amino Acid Composition of Nitrated Albumin and its Cyanogen Bromide Fragments N, M, and C

The results are means of two determinations. The values of tyrosine, 3-nitrotyrosine, and 3,5-dinitrotyrosine are increased by 5%, the remaining values are not corrected. The values found are compared with known data on nonnitrated material, expressed by integral numbers. Tryptophan was not determined. The number of amino acid residues is given.

Amino acid	Designation of material analyzed							
	HSA	HSA	N	N	M	M	C	C
Lysine	59	55·5	9	9·2	19	17·8	31	26·6
Histidine	16	15·5	5	5·2	5	5·0	6	6·8
Arginine	24	24·1	5	5·5	9	9·2	10	10·3
Aspartic acid	53	54·1	16	15·5	14	13·4	23	24·3
Threonine	28	27·6	6	5·8	6	5·6	16	14·8
Serine	24	23·5	3	3·2	9	8·1	12	11·2
Glutamic acid	82	83·3	19	19·0	24	23·2	39	38·8
Proline	24	25·3	5	5·2	5	5·0	14	13·6
Glycine	12	12·1	3	2·9	3	3·0	6	5·8
Alanine	62	62·0	11	11·0	23	23·0	28	28·0
Half-cystine	35	32·6[a]	7	6·0[a]	12	9·8[a]	16	17·0[b]
Valine	41	40·0	10	9·5	5	5·0	26	22·0
Isoleucine	8	7·5	1	0·9	4	3·4	3	3·1
Leucine	61	62·4	12	11·6	19	18·2	30	30·8
Phenylalanine	31	31·0	7	6·8	10	9·8	14	14·6
Tryptophan	1	—	0	—	1	—	0	—
Methionine	6	5·6	2	1·8[c]	1	0·9[c]	3	2·6[c]
3-Nitrotyrosine	—	8·8	—	1·2	—	3·2	—	4·5
3,5-Dinitrotyrosine	—	1·2	—	—	—	0·2	—	1·0
Tyrosine	18	7·1	2	0·6	6	1·8	10	4·0
Total tyrosine	18	17·1	2	1·8	6	5·2	10	9·5

[a] Determined in unoxidized sample; [b] determined as cysteic acid after oxidation; [c] determined as homoserine.

containing c. 1 mg of protein in 1 ml, was digested with chymotrypsin 16 h at 22°C and an enzyme to substrate weight ratio of 1 : 50. The digest was freeze-dried. The tryptic digestion of fragments N and M was performed in a similar manner. For tryptic hydrolysis of fragment C, a 1% solution of the latter in water, made alkaline by 0·1M-NH_4HCO_3 with respect to phenol red as indicator, was digested with trypsin 2 h at 37°C and at an enzyme to substrate weight ratio of 1 : 100. At the end of this period more trypsin was added (to a final ratio of 1 : 50) and the incubation was continued for 2 h. The digest was either directly applied to the column or freeze dried. For combined tryptic-thermolytic hydrolysis 100 mg of fragment C was dissolved in 10 ml of water, the solution was made alkaline by 0·1M-NH_4HCO_3 (containing 1 mM-$CaCl_2$) with respect to phenol red as indicator. The digestion was allowed to proceed at 37°C for 3 h with two successive additions of trypsin (final weight ratio 1 : 50) and for 3 h with two additions of thermolysin (final weight ratio 1 : 50). The digest was lyophilized.

RESULTS

The nitration of native albumin by an excess of tetranitromethane (ratio 4·4 mol of tetranitromethane/mol tyrosine in albumin) afforded nitrated albumin whose degree of nitration of tyrosine residues was determined by amino acid analysis (Table I).

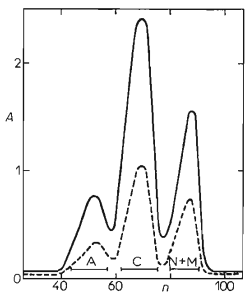


FIG. 2

Elution Profile of Cyanogen Bromide Digest of Nitrated Albumin on Column of Sephadex G-100

The column (100 × 15·5 cm) was eluted by formate buffer, pH 2·8 (2 ml of 26% NH_4OH — 8 ml of 99% formic acid — 990 ml of water). Fractions 150 ml/h, *n* fraction number. Full line absorbance at 280 nm, dashed line absorbance at 356 nm. Pooled fractions A, C, and N + M are marked by bars.

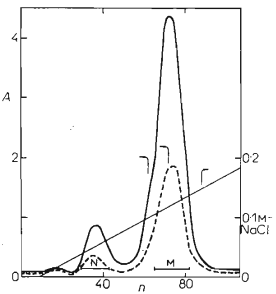


FIG. 3

Elution Profile of Mixture of Fragments N + M on SE-Sephadex C-25

The column (60 × 2·5 cm) was eluted by a linear concentration gradient of 0 to 0·3M-NaCl (total volume 2·6 l) in 50 mM-CH_3COONa buffer containing 8M urea, pH 5·0. Fractions 16·5 ml/10 min, *n* number of fraction. Full line absorbance at 280 nm, dashed line absorbance at 356 nm. Pooled fractions N and M are marked by bars.

It was demonstrated that of the 18 tyrosine residues present in albumin about 7–7·5 remain in original form, 9 are converted into 3-nitrotyrosine, and approximately 1·5 residue to 3,5-dinitrotyrosine[7]. The separation of the cyanogen bromide fragments of nitrated albumin gave an elution profile (Fig. 2 and 3) analogous to that observed with nonnitrated albumin. Unlike with the latter, the high molecular weight part of the digest, contained in peak A, was slightly increased. It was shown in informative experiments that this peak contained aggregated material derived from various regions of the albumin molecule. The identity of cyanogen bromide fragments N, M, and C obtained with the earlier described fragments of albumin, bearing the same designation (Fig. 1), was established also by amino acid analyses (Table I). The nitrated fragments with intact disulfide bonds were subsequently converted into their S-sulfo derivatives and enzymatically digested.

Isolation and Analysis of Nitrotyrosine Peptides of Fragment N

With respect to the information obtained by the analysis of peptide maps of the tryptic and chymotryptic digest of fragment N, chymotrypsin was chosen for preparative-scale digestion experiments. The chymotryptic peptide map of this fragment showed the presence of two main spots containing almost all of the yellow-colored material (Fig. 4*a*). In the preparative experiment 70 mg of the digest was separated electrophoretically at pH 5·6. Two yellow bands were obtained. The nitrotyrosine peptides contained in this material were purified by paper chromatography and characterized by amino acid analysis (Table II). On the basis of the knowledge of the amino acid composition of the peptides, the complete amino acid sequence of human serum albumin[1,2], and with respect to the specificity of the enzyme used for the cleavage (chymotrypsin in the case of fragment N), we were able, in this as well as in the other cases, to unambiguously identify the positions of the peptides isolated and of the tyrosine residues in the structure of albumin. The peptides derived from fragment N were marked N—Cl and N–C2.

Peptide N—Cl (Table II) corresponds to the sequential region ...Ala-Gln-Tyr-.., involving the tyrosine residue which occupies position No 30 in albumin. Peptide N–C2 corresponds to the region ..Arg-Glu-Thr-Tyr.., involving Tyr(84). Both tyrosine residue of fragment N, No 30 and 84 were found in nitrated form.

Isolation and Analysis of Nitrotyrosine Peptides of Fragment M

Fragment M (210 mg) was digested with chymotrypsin. The nitrotyrosine peptides M–Cl to M–C5 (Fig. 4*b*) were isolated by electrophoresis and paper chromatography. From the knowledge of their amino acid composition (Table II) these peptides were unambiguously allocated to the albumin structure.

TABLE II

Amino Acid Composition of Nitrotyrosine Peptides

The peptides were isolated from cyanogen bromide fragments N, M, and C digested with chymotrypsin (C) or trypsin and thermolysin (TTh). Homoserine and tryptophan were not determined. The number of amino acid residue is given.

Amino acid	Designation of peptide												
	N—C1	N—C2	M—C1	M—C2	M—C3	M—C4	M—C5	C—TTh1	C—TTh1′	C—TTh2	C—TTh3	C—TTh4	C—TTh5	C—TTh6
Lysine			2·2			1·3	1·8				0·9	1·0		
Histidine				0·9						0·9				
Arginine		1·0		2·0		1·0	1·0		1·1	1·0				
Aspartic acid										1·0			1·0	0·9
Threonine		0·8										1·0		1·2
Serine										1·0				
Glutamic acid	1·0	1·0		1·1	1·0			1·0			0·9		1·0	1·9
Proline				0·8	1·0					0·8			1·0	
Glycine											1·0			
Alanine	1·0			1·0	1·0	0·8	3·0		1·0				1·0	
Half-cystine[a]													0·9	
Valine														0·9
Isoleucine				0·8										
Leucine			1·0		2·1			1·0			1·2			1·0
Phenylalanine					0·8		0·8							
Tyrosine								0·9						
3-Nitrotyrosine	0·8	0·8	1·0	0·8	0·8	0·8		0·9	0·8	1·1	0·7		0·8	0·8
3,5-Dinitrotyrosine							0·7					1·0		

[a] Determined as cysteic acid after oxidation.

Peptide M – C1 corresponds to the sequential region of albumin ..Leu-Lys-Lys--Tyr.., containing tyrosine residue No 138. *Peptide M – C2* comprizes the region ..Glu-Ile-Ala-Arg-Arg-His-Pro-Tyr.., C-terminated with Tyr(148). *Peptide M – C3* has the same amino acid composition as peptide "C4" isolated earlier[19] from the chymotryptic digest of fragment CB3(Cys). It corresponds therefore to the region ..Phe-Tyr-Ala-Pro-Glu-Leu-Leu.., involving Tyr(150). *Peptide M – C4* corresponds to the region ..Ala-Lys-Arg-Tyr.. containing tyrosine No 161. *Peptide M – C5*, corresponding to the sequence ..Ala-Lys-Arg-Tyr-Lys-Ala-Ala-Phe.. is longer than M – C4 yet contains also Tyr(161). The tyrosine residue in this peptide was nitrated to 3,5-dinitrotyrosine. Hence, the tyrosine residues No 138, 148, 150, and 161 of fragment M were found in nitrated form.

Isolation and Analysis of Nitrotyrosine Peptides of Fragment C

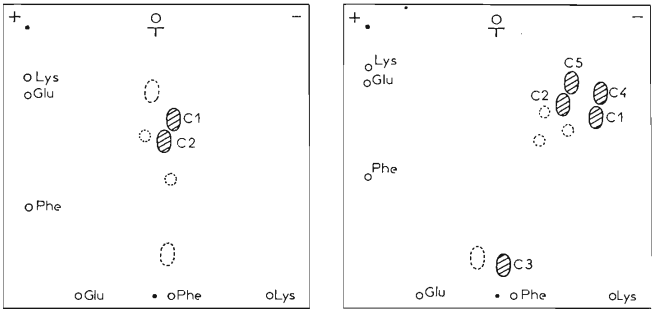
The character and distribution of spots on the chymotryptic peptide map of this fragment showed that chymotryptic digestion was unsuitable. Therefore combined hydrolysis with trypsin and thermolysin carried out stepwise was chosen for preparative-scale isolation of nitrotyrosine peptides. This type of digestion yielded well-defined spots separable by paper techniques. The first separation of 65 mg of the digest was carried out by two-dimensional peptide maps (Fig. 4*c*) permitting the isolation of all peptides by a uniform procedure. The corresponding yellow spots were cut out and eluted together. The nitrotyrosine peptides eluted were purified by paper electrophoresis at pH 1·9 and paper chromatography. Peptides C – TTh1 to C – TTh6 were isolated and characterized.

Peptide C – TTh1 is derived from the sequential region ..Leu-Tyr-Glu-Tyr.. containing tyrosine residues No 332 and 334 of albumin. From the data of amino acid analysis (Table II) only one of the two tyrosine residues is nitrated, yet it was not determined which one. In the informative experiments described above, a small quantity of peptide C – TTh1′ (Table II), corresponding to the region ..Tyr-Ala-Arg.. and containing Tyr (334) was isolated in addition to peptide C – TTh1. We assume from this complementary information that residues No 334 in peptide C – TTh1 is nitrated. *Peptide C – TTh2* corresponds to albumin sequence ..Arg-His-Pro-Asp-Tyr-Ser-.. containing Tyr(341). *Peptide C – TTh3* from the region ..Leu-Gly-Glu-Tyr-Lys.. contains Tyr(401). *Peptide C – TTh4* contains Tyr(411) in the sequence ..Tyr-Thr--Lys.. This residue was nitrated to 3,5-dinitrotyrosine. *Peptide C – TTh5* corresponds to the region ..Pro-Cys-Ala-Glu-Asp-Tyr.., involving Tyr(452). *Peptide C – TTh6* is identical with the region ..Leu-Glu-Val-Asp-Glu-Thr-Tyr.. in which Tyr(497) is present. The tyrosine residues No 334, 341, 401, 411, 452, and 497 of fragment C were found in nitrated form.

Semiquantitative Determination of Individual Nitrotyrosine Residues

An exact quantitative determination of the degree of nitration of each individual nitrotyrosine residue would require the estimation of individual yield coefficients for each peptide and each purification step. Because of the high number of tyrosine residues investigated such procedure would be subject to considerable experimental error; therefore we evaluated the nitrotyrosine residues at a semiquantitative level.

The yellow peptides (Fig. 4*a*–4*c*) were isolated from the enzymic digest of each fragment; these peptides represented 90% of the nitrotyrosine material present.



a *b* *c*

FIG. 4

Peptide Maps of Cyanogen Bromide Fragments of Nitrated Albumin

First direction (horizontally) high voltage electrophoresis at pH 5·6. Second direction (vertically) descending chromatography. ○ origin, ● reference mixture of amino acids (Lys,Glu,Phe). The nitrotyrosine peptides identified are marked by hatching, the positions of weak nitrotyrosine peptides are outlined by dashed lines. *a* peptides N—Cl to N-C2 (the abbreviated designation is used) from the chymotryptic digest of fragment N. *b* peptides M—Cl to M—C5 from chymotryptic digest of fragment M. *c* peptides C-TTh1 to C-TTh6 from tryptic-thermolytic digest of fragment C.

From the amino acid composition of the peptides their positions in albumin were determined. The total nitrotyrosine yield in the individual peptides was determined by amino acid analysis. The knowledge of the total nitrotyrosine content of each fragment (Table I) then permits a semiquantitative evaluation of the individual tyrosine residues to be carried out.

The quantity of 3-nitrotyrosine (or 3,5-dinitrotyrosine) found in the peptide was multiplied by a mean correction coefficient c calculated from the formula

$$c = 0{\cdot}9 \,.\, F/P \,,$$

where the number 0·9 expresses the amount of nitrotyrosine material accounted for by the peptides isolated, F the total number of mol of 3-nitrotyrosine and 3,5-dinitrotyrosine per mol of cyanogen bromide fragment, and P the sum of the two nitrotyrosine forms in the peptides analyzed and derived from 1 mol of the fragment given. The mean correction coefficient for fragment N is 1·20, for fragment M 1·28, and for

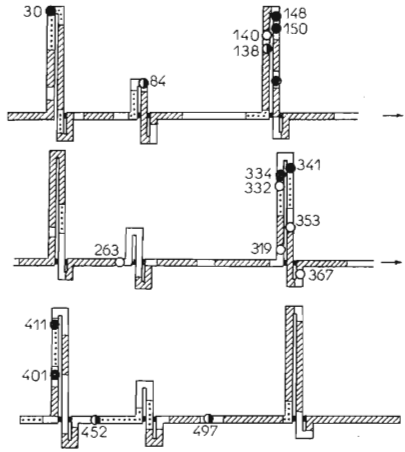


FIG. 5

Schematic Representation of Steric Accessibility of Tyrosine Residues to Nitrating Agent in Hypothetical Secondary Structure of Human Serum Albumin

● — Strongly nitrated tyrosine residues, ◑ — medium-nitrated residues, ○ — residues not detected in nitrated form. The helical regions of the polypeptide chain are marked by hatching, beta-structures by dotting. Regions of unorganized structure are not marked.

fragment C 1·43. The coefficients increase with the increasing molecular weight of the fragments and complexity of the digests fractionated. Since we used mean coefficients the results obtained are rather of comparative value. Therefore, according to the final evaluation, the tyrosine residues were divided into three categories (Table III): strongly nitrated (corrected values above 0·6), medium nitrated (corrected values up to 0·6), and residues not found in nitrotyrosine peptides. The final result characterizing the topography of the tyrosine residues in albumin is shown in Fig. 5. The schematic representation of the albumin molecule and its disulfide bonds also shows, as complementary information, regions of the hypothetic secondary structure of albumin[20] calculated from its amino acid sequence by using the empirical rules[21].

TABLE III

Semiquantitative Evaluation of Nitration of Individual Tyrosine Residues in Human Serum Albumin

Tyrosine No	Peptide	Quantity of peptide mol/mol fragment	Corrected content of nitrotyrosine mol/mol fragment	Reaction of tyrosine residue with tetranitromethane
30	N—C1	0·60	0·7	strong
84	N—C2	0·30	0·4	medium
138	M—C1	0·39	0·5	medium
140	—	—	—	not found
148	M—C2	0·55	0·7	strong
150	M—C3	0·70	0·9	strong
161	M—C4	0·60	0·8	strong[a]
161	M—C5	0·15[b]	0·2	strong[a]
263	—	—	—	not found
319	—	—	—	not found
332	—	—	—	not found
334	C—TTh1	0·70	1·0	strong
341	C—TTh2	0·60	0·9	strong
353	—	—	—	not found
367	—	—	—	not found
401	C—TTh3	0·64	0·9	strong
411	C—TTh4	0·67[b]	1·0	strong
452	C—TTh5	0·41	0·6	medium
497	C—TTh6	0·43	0·6	medium

[a] The sum in M—C4 and M—C5 is 1·0; [b] determined as 3,5-dinitrotyrosine.

DISCUSSION

The present state of our knowledge of the primary structure of human serum albumin and the arrangement of its polypeptide chain in double disulfide loops pave the way toward the elucidation of problems of the sterical arrangement of the molecules in domains whose hypothetical model has been proposed by Foster twenty years ago[5]. One of the lines of approach to the characterization of the sterical parameters of the molecule represent studies on the topography of tyrosine residues based on their accessibility to the reaction with tetranitromethane[7]. The eighteen tyrosine residues of human serum albumin are distributed relatively uniformly along its chain (Fig. 1). Malan and Edelhoch[9], studying the nitration of tyrosine residues in human serum albumin, carried out the reaction with an excess of tetranitromethane at pH 8 which is convenient for the course of the reaction[7]. They have shown that only 50% (8·7) of the tyrosine residues present in the molecule of HSA can be nitrated; this number was approximately the same when the reaction was carried out in 8M urea. In this study we chose the same conditions of nitration, *i.e.* pH 8·0 and an excess of 80 mol of tetranitromethane per mol of HSA (4·4 mol tetranitromethane/mol Tyr), permitting a comparison of the results. The results obtained therefore characterize the reactivity of the individual tyrosine residues at pH 8·0. The pH-dependent conformational changes of the albumin molecule can lead of course to differences in the exposition of certain tyrosine residues under different conditions.

The content of 8·8 residues of 3-nitrotyrosine found in nitrated HSA by amino acid analysis, permitting an easy determination of the nitrated products of tyrosine, is in good agreement with the data obtained by other authors[9]; however, an additional 1·2 residue was detected in the form of 3,5-dinitrotyrosine. When the cyanogen bromide digest of nitrated albumin was fractionated an increased amount of the aggregated cleavage products was observed (Fig. 2), the elution profile, however, was essentially the same as that obtained with nonnitrated albumin[8] and the corresponding nitrated cyanogen bromide fragments N, M, and C were obtained. The degree of nitration of each albumin fragment was determined by amino acid analysis (Table I) which confirmed the identity of the fragments. Following the semiquantitative evaluation (Table III) all tyrosine residues of HSA were classified as falling into three groups, *i.e.* strongly nitrated (exposed on the surface of the molecule), medium nitrated (occupying positions exposed to a limited degree), and inaccessible to tetranitromethane, which were not found in nitrated form.

In Fig. 5 the positions of the tyrosine residues are shown in a schematic model of the albumin molecule with 9 double loops in 3 homologous regions. As an important (yet not the only) factor essential for the accessibility of the tyrosine residues appears their distance from disulfide bonds, closely connecting the regions of the chain in double loops. None of the most exposed tyrosines is localized in the neighbor-

hood of disulfides; by contrast, the residues occupying such positions, *i.e.* No 263, 319, 367, and the somewhat more distant residue No 353 are inaccessible to tetranitromethane. The positions of residues No 84, 452, and 497 are also in accordance with their lower accessibility. Residues No 30, 148, 150, 334, 341, and 411 placed on the most distant tops of the long disulfide loops belong to the most exposed ones. Residue No 401 is also exposed. The difference in the behavior of residues No 138, 140, and 332 indicates the limiting effect of other conformational factors. The scheme in Fig. 5 presents by way of complementary information also the regions of the hypothetical secondary structure of albumin[20] calculated from sequential data by using empirical rules[21]. According to these rules tyrosine behaves as a helix breaker and does not occur in any of the helical regions. The scheme shows the differences in the behavior upon nitration between residues No 138, 140, 161, and residues No 332, 334, and 353 occupying homologous positions. The different accessibility of these residues is in accordance with differences in the arrangement of these regions of the hypothetical secondary structure. This indicates a differentiation in the homologous architecture of the albumin domains. To numerous factors limiting the accessibility of tyrosines in albumin also belongs the existence of more complicated formations of the tertiary structure[5,22] originating in the interaction of the ordered regions of the chain.

The finding of 1·2 residue of 3,5-dinitrotyrosine in nitrated albumin is somewhat surprising. Malan and Edelhoch[9] nitrated in their model experiments free tyrosine and found only traces of this nitration product when a ten-fold molar excess of tetranitromethane was used. The finding of 1·2 residue of 3,5-dinitrotyrosine leads us to assume that the efficiency of the reagent was increased compared to the reactivity of free tyrosine. As 3,5-dinitrotyrosine were detected the strongly reactive residue No 411 (Table III) and 0·2 residue No 161, occurring predominantly in the form of 3-nitrotyrosine. Shaw[23] studied the reaction of certain enzymes and biological preparations with radioactive diisopropylfluorophosphate and observed the labeling of one tyrosine residue only in rabbit and human serum albumin. We have assigned in earlier work[24] this reactive tyrosine residue to fragment CB5(Phe) of human serum albumin, *i.e.* to position No 411. The nearest neighborhood of both anomalous tyrosines is ..Ala-$\overset{161}{\text{Lys}}$-Arg-Tyr-Lys-Ala.. and ..Val-$\overset{411}{\text{Arg}}$-Tyr-Thr-Lys-Lys.. and is characterized by a cumulation of basic amino acid residues. If we compare the positions of these residues in Fig. 5 we observe that residue No 411 is moreover localized at an exposed site close to the top of the long disulfide loop. The increased reactivity of these tyrosines is obviously affected both by the nearest neighborhood and also by overall topographic factors due either to the effect of intramolecular catalysis or to binding or local concentration of acidic reagents or reaction intermediates.

The results of our first topographic study comply with the conclusion that the formation of primary binding centers proceeds with the participation of the exposed

tops of the disulfide loops which are sufficiently sterically accessible whereas the compact unaccessible region around the disulfide bonds stabilizes the characteristic architecture of the albumin molecule.

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