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Topography of Human Plasma α_1-Acid Glycoprotein†

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ABSTRACT: Human plasma α_1-acid glycoprotein, whose linear amino acid sequence has recently been elucidated (Schmid et al. (1973), *Biochemistry 12,* 2711), was further investigated with regard to its topography. Nitration of this protein and subsequent elucidation of the structures of the peptides containing a modified tyrosine indicated that residues 27, 37, 78, 115, 127, and 157 are free, 50 and 91 are in an intermediate state, and 65, 74, 110, and 142 are buried. CD measurements between pH 10 and 12 demonstrated that the buried tyrosines are strongly hydrogen bonded and are probably responsible to a considerable extent for the stability of this protein. Of the three tryptophans of this protein, residue 122 proved to be partially reactive with Koshland reagent while the other two (25 and 160) were found to be unreactive. The state of the two disulfide bonds, established by differential reduction and alkylation with specific reagents, was shown to be of an intermediate type. Using carboxymethylation with bromoacetate at pH 7.0 for 8 days, the three histidines (97, 100, and 171) and methionine 111 could be shown to be in intermediate states. All lysines were treated with trinitrobenzenesulfonate and thus were assumed to be free. Of the 40 carboxylic groups, which were amidated with glycine methyl ester, 32 including the 14 sialyl residues were found to be free, six in an intermediate and the remaining two in a buried state. The present study describes the states of almost half of the amino acid residues of α_1-acid glycoprotein, a knowledge important for the construction of a preliminary three-dimensional model of this conjugated protein.

The complete amino acid sequence of α_1-acid glycoprotein, one of the most extensively studied human plasma proteins (Jeanloz, 1972), has recently been established (Ikenaka et al., 1972; Schmid et al., 1973). However, the three-dimensional structure of this globulin still remains unknown. An investigation on the topography of this conjugated protein was initiated earlier in order to elucidate certain aspects of the complete structure of this protein. Yamagami and Schmid (1967) and Yamagami et al. (1968) determined the number of "free[1]" and "buried[1]" tryptophan and tyrosine residues of this globulin.

The present paper describes an extension of this topographical investigation, the determination of the state of the carboxylic, cystine, histidine, lysine, and methionine residues. In addition, the exact locations of the free, intermediate, and buried tryptophan and tyrosine residues within the primary structure of this protein were also established.

Materials and Methods

α_1-Acid Glycoprotein. This protein was isolated from the supernatant solution of Cohn fraction V derived from pooled normal human plasma (Cohn et al., 1946) by a method previously described (Bürgi and Schmid, 1961). The homogeneity of this globulin was established by several criteria of purity (Schmid, 1976; Ikenaka et al., 1966). It should be emphasized that, throughout this study, pooled α_1-acid glycoprotein was utilized as starting material which possesses 22 amino acid substitutions (Schmid et al., 1973) and five carbohydrate units with 15 sialyl residues.

Reagents. Pepsin, three times crystallized, and chymotrypsin, two times crystallized, were obtained from Worthington Biochemical Corp., Freehold, N.J. The following special reagents were purchased; HNBB[2] and α-amino-L-butyric acid methyl ester hydrochloride from Cyclo Chemical Co., Los Angeles, Calif.; TNM, 2,4,6-trinitrobenzenesulfonic acid and 1-fluoro-2,4-dinitrobenzene and bromoacetic acid which was recrystallized from cyclohexanone, from Eastman Organic Chemicals Co., Rochester, N.Y.; EDC from Ott Chemical Co.,

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[1] The terms "free", "intermediate", and "buried" are used as defined by Riordan et al. (1965) and Yamagami and Schmid (1967). Hence, a free lysine, for example, is a lysine residue of a native protein whose ε-amino group undergoes chemical reactions with specific reagents under mild conditions.

[2] Abbreviations used are grouped into chemical (AEL, aminoethyl; CM, carboxymethyl; Dnp, dinitrophenyl; EDC, 1-ethyl-ε-(3-dimethylaminopropyl)carbodiimide; HNBB, 2-hydroxy-5-nitrobenzyl bromide; TNM, tetranitromethane; Tnp, trinitrophenyl) and technical designations (CD, circular dichroism; CNBr-I, CNBr-II, CNBr-III, and CNBr-IV, cyanogen bromide fragments I, II, III, and IV, respectively; ND, not determined).

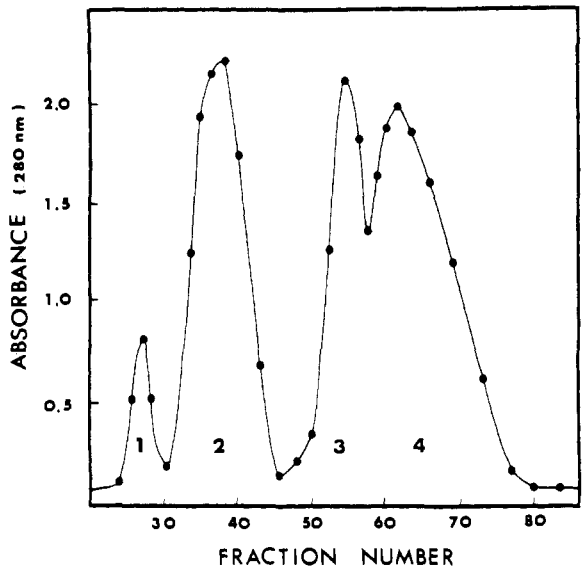

FIGURE 1: Fractionation of a chymotryptic digest of CNBr-I derived from nitrated α_1-acid glycoprotein. The gel filtration through Sephadex G-50 column (2 × 100 cm, 0.2 M acetic acid) was carried out at a flow rate of 20 ml/h, and 5 ml of the effluent per test tube was collected. The effluent was monitored by its absorbance at 280 nm. The first fraction contained aggregated proteinaceous material which was not further studied. Fractions 2 to 4 were further investigated as described in the text.

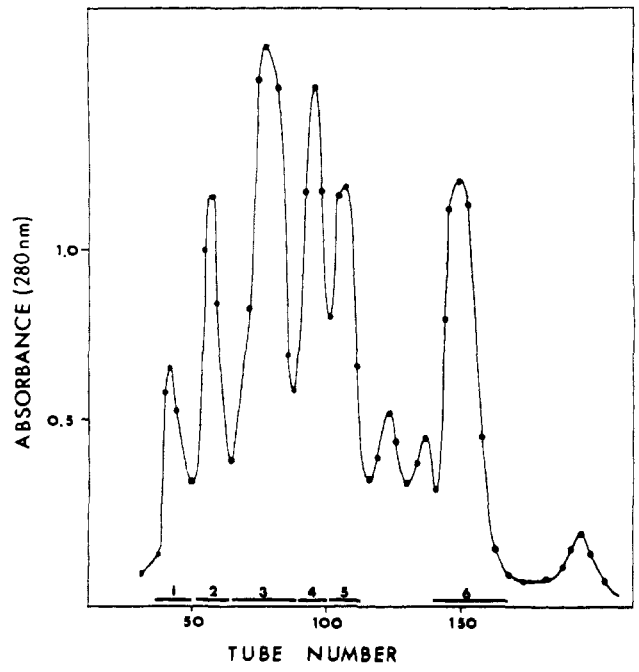

FIGURE 2: Fractionation of a chymotryptic digest of CNBr-II derived from nitrated α_1-acid glycoprotein. The gel filtration through a Sephadex G-25 column (1.4 × 160 cm, 0.2 M acetic acid) was carried out at a flow rate of 5.0 ml/h and 3.0 ml of the effluent per test tube was collected. The effluent was monitored by its absorbance at 280 nm. For further details, see text.

Muskegan, Mich.; glycine methyl ester hydrochloride from K & K Laboratories, Inc., Plainview, N.Y., and Pronase (70 000 puk/g) from Kaken Kagaku, Inc., Tokyo, Japan.

Nitration of the Tyrosine Residues. α_1-Acid glycoprotein (0.8 g) was nitrated according to Riordan et al. (1967) as described by Yamagami et al. (1968). Excess reagent was removed by Sephadex G-25 chromatography.[3] The number of nitrotyrosine residues was calculated from the molar absorbance at 428 nm of the modified protein and the molar absorbance of 4200 of nitrotyrosine at pH 9 (Yamagami et al., 1968). The number of modified residues was also determined from the amino acid composition of the nitrated protein using a Technicon analyzer (0.6 × 160 cm column). In order to prevent destruction of tyrosine and its derivatives, 2 μl of water saturated with phenol (Sanger and Thompson, 1963) was added to the 1.0 ml of constant boiling HCl used for total hydrolysis of the modified protein (110 °C, 24 h, N_2, sealed tube). 3,5-Dinitrotyrosine was eluted after valine (Sairam et al., 1972) and 3-nitrotyrosine immediately after phenylalanine on the mentioned autoanalyzer (Sokolovsky et al., 1970).

Isolation of Peptides Containing Nitrotyrosine Residues. α_1-Acid glycoprotein (0.8 g), nitrated as indicated above, was treated with CNBr as described earlier (Schmid et al., 1973). After reduction and alkylation, the resulting four fragments (CNBr-I, CNBr-II, CNBr-III and CNBr-IV) were separated from each other by chromatography on a Sephadex G-100 column (Ikenaka et al., 1972). Modified tyrosine residues were found to be present in all fragments as judged by their yellow color exhibited at alkaline pH. CNBr-I and -II were digested with chymotrypsin under conventional conditions (Schmid et al., 1973). The nitrotyrosine-containing peptides of the digest of CNBr-I were isolated as follows. Gel filtration yielded four fractions (Figure 1) of which only fractions 2 and 4 possessed nitro groups. Fraction 2 containing the glycopeptides was desialyzed (pH 1.8, 1 h, 80 °C), neutralized, desalted, and subjected to preparative high voltage electrophoresis at pH 6.5 (4 kV, 85 mA, 5 h, pyridine–acetate buffer, Whatman No. 3MM). Seven nitrotyrosine-containing subfractions were obtained which were then chromatographed on CM-cellulose columns yielding glycopeptides G-Y1, G-Y3, G-Y6, G-Y8, G-Y9, and G-Y10 (Table I). Fraction 4 was subjected to peptide mapping (Nimberg et al., 1971) and three nitrotyrosine-containing peptides (Y2, Y4, and Y5) were obtained. The modified peptides derived from the chymotryptic digest of CNBr-II were isolated as follows. Gel filtration afforded six fractions (Figure 2). Fractions 2, 3, 4, and 6 contained modified peptides (Y11, Y12, Y13, Y14, and Y15) which were subsequently isolated by the mentioned peptide mapping procedure.

The *purity of the peptides* was established by high voltage electrophoresis at two different pH values (6.5, 1.8, and/or 9.5), by paper chromatography (Schmid et al., 1973), and by determination of the amino-terminal amino acids by either the dansyl procedure (Gray, 1972) or the direct Edman technique (Schmid et al., 1973). It is of interest to note that, on two-dimensional chromatography on polyamide sheets (Woods and Wang, 1967), dansyl-3-nitrotyrosine migrated just ahead of bis(dansyllysine) in the second dimension (benzene–acetic acid, 9:1), while it was almost stationary in the first dimension (1.5% formic acid). The *amino acid compositions* of the glycopeptides were determined by the above mentioned autoanalyzer, whereas those of the peptides were measured by the Jeolco autoanalyzer. The presence of *hexose* in the various fractions was assessed by either the orcinol or anthrone procedure (Spiro, 1966).

Substitution of the Tryptophan Residues with HNBB. α_1-Acid glycoprotein (0.8 g) was treated with HNBB according to Horton and Koshland (1972) as described by Yamagami et al. (1968). The number of modified tryptophan residues was determined from the optical density at 410 nm, and by measuring the number of unreacted tryptophan residues

[3] If the pH value of the solvent used for the Sephadex G-25 chromatography for the removal of excess reagents was 8.0, high molecular weight aggregates were formed, agreeing with the observation reported earlier by Sokolovsky et al. (1970).

Table 1: Amino Acid Compositions of Peptides and Glycopeptides[a] with Nitrated Tyrosine.

AA[d]	G-Y1	Y2	G-Y3	Y4	Y5	G-Y6	G-Y7	G-Y8	G-Y9	G-Y10	Y11	Y12	Y13	Y14	Y15
Asp	2.4 (2)	1.4 (1)	2.0 (2)			1.9 (2)	1.0 (1)	1.5 (2)	2.1 (2)	1.0 (1)		3.2 (4)	0.9 (1)	1.0 (1)	0.9 (1)
Thr	3.0 (3)		0.9 (1)			2.5 (3)	1.9 (2)	1.0 (1)	1.2 (1)	1.0 (1)		1.0 (1)	1.0 (1)	0.5 (2)	0.7 (1)
Ser	1.0 (1)		0.9 (1)					0.9 (1)	1.9 (2)	0.7 (1)	0.9 (1)	0.6 (1)	0.3 (1)	0.9 (1)	0.9 (1)
Glu	1.0 (1)	2.0 (2)	4.0 (5)			1.1 (1)		1.8 (2)	2.3 (2)	2.7 (3)		2.2 (2)	2.0 (1)	5.1 (4)	
Pro	2.8 (2)					1.0 (1)						0.9 (1)	0.7 (1)	0.9 (1)	1.0 (1)
Gly	0.4 (1)								1.5 (1)	2.1 (3)	1.0 (1)	0.7 (1)	0.9 (1)	1.0 (1)	
Ala	2.4 (3)		0.5 (1)							0.7 (1)		1.0 (1)	0.7 (1)		
Val	2.0 (2)		0.5 (1)					1.0 (1)	2.5 (2)			0.8 (1)	0.7 (1)		1.6 (2)
Ile	1.7 (3)		0.7 (1)			1.0 (1)				0.7 (1)					0.9 (1)
Leu	2.2 (2)					0.7 (1)	0.5 (1)	0.8 (1)	1.0 (1)	0.7 (1)	0.9 (1)	1.3 (1)	0.8 (1)	0.9 (1)	
Tyr						0.6[b]				0.4[c]					
Phe	0.4 (1)		0.4 (1)	2.0 (2)	1.0 (1)	1.7 (2)				0.7 (1)				0.3 (1)	
3-Nitro-Tyr	0.7 (1)	0.8 (1)[b]	1.0 (1)	0.8 (1)	0.9 (1)	0.4[b]	0.7 (1)	1.0 (1)	0.8 (1)	0.4[c]	0.9 (1)	0.8 (1)	0.8 (1)	0.9 (1)	0.7 (1)
Lys	0.6 (1)		1.1 (1)			0.9 (1)		1.0 (1)				1.6 (2)	1.0 (1)	2.3 (2)	1.0 (1)
His										2.4 (2)					
Arg		0.7 (1)	1.0 (1)					1.0 (1)	0.9 (1)	0.3 (1)					0.8 (1)
Trp	ND (1)											ND (1)			
No. of R[e]	24	5	16	3	2	13	5	11	14	17	4	18	11	15	10
Yield (%)	52	22	28	6	14	5.2	13	14	9	6	15	22	14	11	63
Position in sequence	8–31	32–37	32–47	49–51	50–51	50–62	75–79	75–84	75–88	85–101	113–116	116–133	123–133	125–140	149–158

[a] The glycopeptides are designated with the prefix G. [b,c] The sum of tyrosine and 3-nitrotyrosine is approximately 1. [d] AA, amino acid. [e] R, residues.

with the aid of an autoanalyzer after hydrolysis of the modified protein with 5 N NaOH (Hugli and Moore, 1972). In a control experiment the number of tryptophan residues of the native α_1-acid glycoprotein was also determined.

Isolation of Peptides with HNBB–Tryptophan. α_1-Acid glycoprotein (0.8 g) modified with HNBB[4] was digested with pepsin (pH 2.0, 37 °C, 3 h, 1% substrate, enzyme–substrate ratio 1:100) and passed through a Sephadex column yielding three fractions. Three additional fractions were obtained by applying a gradient in the acetic acid concentration of from 5 to 30%. Each fraction was subjected to the mentioned peptide mapping technique, and the HNB–peptides were located as yellow spots when NH_3 vapors were passed over the dry map. Moreover, on Ehrlich's stain the modified peptides appeared as gray–purple, whereas the peptides with unmodified tryptophan residues appeared as purple spots (Barman and Koshland, 1967). Each peptide was eluted with 5% acetic acid and then with water followed with 1% ammonium hydroxide solution, and each extract was lyophilized separately. The purity and composition of these peptides were established as indicated above.

Reduction of the Disulfide Bonds. α_1-Acid glycoprotein was reduced under mild conditions (Ishiguro et al., 1969) and the formed sulfhydryl groups were substituted using ethyleneamine (Cole, 1967). The modified protein was then completely reduced under stronger conditions in the presence of 6 M urea (Ishiguro et al., 1969; Ikenaka et al., 1972) and the newly formed sulfhydryl groups were now alkylated using iodoacetate. The resulting protein preparation was next treated with CNBr, and the four formed fragments were separated from each other (Ikenaka et al., 1972) and then subjected to amino acid analysis. *S*-(β-Aminoethyl)cysteine was eluted immediately after lysine (Raftery and Cole, 1966).

Carboxymethylation of the Methionines and Histidines. α_1-Acid glycoprotein (0.2 g) was treated with bromoacetate at pH 7.0 for a period of 8 days according to the method of Gurd (1972), Nigen and Gurd (1973), and Castellino and Hill (1970). Appropriate aliquots of the reaction mixture were removed at various intervals, separated from the excess reagents by gel filtration, lyophilized, and hydrolyzed with constant boiling HCl as indicated above. The numbers of unmodified methionine and histidine residues were obtained from the amino acid composition of the modified protein. For the carboxymethylation of all methionine and the largest part of the histidine residues, the glycoprotein was treated in the same manner but in the presence of 6 M urea.

Trinitrophenylation of the Lysines. α_1-Acid glycoprotein was first trinitrophenylated with 2,4,6-trinitrobenzenesulfonic acid at pH 8.0 (Satake et al., 1960). Aliquots of the reaction mixture (0.05 μM) were removed after various periods of reaction. In each case the reaction was terminated by lowering the pH to 2.5 with 1 N HCl. The number of introduced Tnp residues was determined spectrophotometrically (Satake et al., 1960).

In an additional experiment, after a reaction period of 2 h the trinitrophenylated protein (0.2 μM) was desalted and lyophilized. Subsequently, as the second substitution reaction, the trinitrophenylated protein was now dinitrophenylated. The resulting preparation was hydrolyzed, and the hydrolysate dissolved in 1 N HCl was applied to a talcum column (2 × 10 cm) (Sanger, 1949) which retained both the ϵ-Tnp and ϵ-Dnp-lysines. These two lysine derivatives were eluted from

[4] If HNBB-α_1-acid glycoprotein was treated with CNBr followed by reduction and carboxymethylation, a gel resulted.

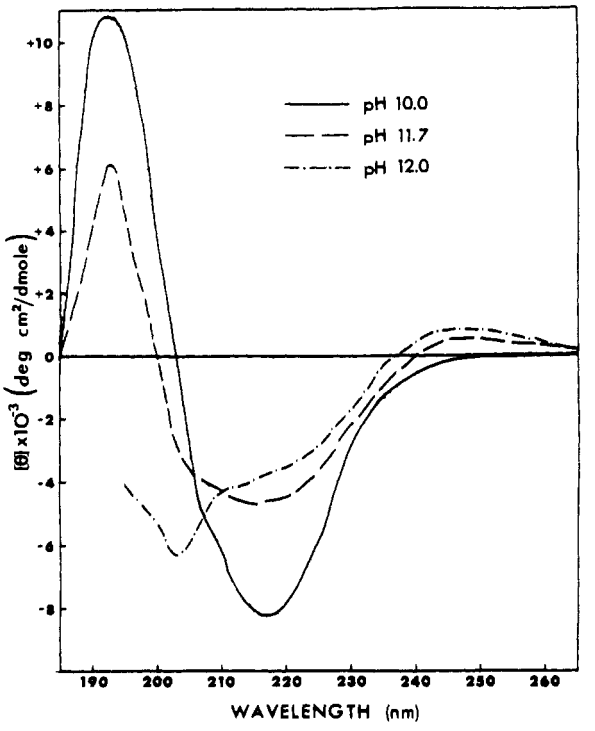

FIGURE 3: Circular dichroism of native α_1-acid glycoprotein between pH 10 and 12 in $\Gamma/2$ 0.02 potassium phosphate buffers.

the column with acidified ethanol, dinitrophenylated, and resolved by two-dimensional paper chromatography (Frankel-Conrat et al., 1955). Further, the native protein was dinitrophenylated, without prior trinitrophenylation, hydrolyzed, and chromatographed as described above to assess the effect of the solvent (66% ethanol) on the dinitrophenylation reaction.

Amidation of the Carboxylic Groups. The carboxylic groups of α_1-acid glycoprotein (0.2 g) were treated with glycine methyl ester in the presence of EDC at pH 4.75 (Hoare and Koshland, 1967). Aliquots of the reaction mixture, withdrawn after various intervals, were passed through a Sephadex G-25 column to remove excess reagent and salt. The number of the carboxylic groups remaining unmodified after 4 h was determined by treating this modified protein by a similar procedure but in the presence of 8 M urea and utilizing α-aminobutyric methyl ester as the nucleophilic reagent instead of glycine methyl ester. The resulting double-labeled protein preparation was hydrolyzed, and the amount of α-aminobutyric acid was measured by an autoanalyzer.

Circular Dichroism. The circular dichroism of α_1-acid glycoprotein and that of several derivatives were measured with the aid of a Cary Model 61 spectropolarimeter. Measurements were made at 25 °C between 250 and 185 nm (0.1-cm cell). The protein concentration, varying between 0.1 and 0.5 mg per ml of solvent, was determined spectrophotometrically using the $E_{280}^{1\%}$ value of 8.93 (Schmid, 1953) or, in the case of the Tnp-protein, by weight assuming 7% of the preparation to be salt and water (average value of three determinations). Unless otherwise stated, all preparations were dissolved in water. For the calculation of the mean residue ellipticity, the mean residue weight for the polypeptide moiety of the glycoprotein was calculated to be 117. The carbohydrate moiety was not included in this value, because the glycopeptides derived from α_1-acid glycoprotein essentially did not contribute to the CD of the protein. The mean residue weight of a glycopeptide fraction derived from a chymotryptic digest of α_1-acid glycoprotein used as control was 180.

Additional Procedures. Horizontal starch gel electrophoresis was carried out in pH 8.6, $\Gamma/2$ 0.1 diethyl barbiturate-citrate buffer. For the determination of the sedimentation coefficient a Spinco Model E ultracentrifuge equipped with Schlieren optics and a rotor temperature control unit (RTIC) was utilized.

Results and Discussion

The State of the Tyrosine Residues. The Number of Nitrated Tyrosine Residues. Nitration of α_1-acid glycoprotein revealed that six (6.2) residues could be modified as judged by spectrophotometric determinations. Amino acid analysis of the modified protein also indicated that the same number of residues (6.5) was modified and that only the monosubstituted tyrosine was obtained. Amino acid analyses of the CNBr fragments of nitrated α_1-acid glycoprotein revealed that 4 of the 7.5 tyrosine residues of CNBr-I and 2.5 of the 3.5 tyrosine residues of CNBr-II were modified. CNBr-III and CNBr-IV contained 1.5 and 1.0 modified residues.

The Amino Acid Sequences of the Peptides with Nitrated Tyrosine Residues and the Location of These Residues in the Amino Acid Sequence of the Protein. The amino acid compositions of the glycopeptides and peptides with tyrosine residues isolated from a chymotryptic digest of CNBr-I are listed in Table I. Of particular interest are the two peptides G-Y6 and G-Y10 which contained both tyrosine and nitrotyrosine and accounted together for 1 mol in each compound indicating partial nitration. The sum of the molar recovery of the peptides which contained the same tyrosine residues varied between 25 and 50%. The elucidated amino acid sequences of these peptides are listed in Table II. These sequences were established on the basis of the amino acid compositions and the amino-terminal sequences of the peptides and a comparison with the complete amino acid sequence of the glycoprotein established earlier (Schmid et al., 1973).

The States of the Tyrosine Residues. The states of the 12 tyrosine residues were found to be as follows. Five tyrosines (residues 27, 37, 78, 127, and 157) and one tyrosine replacement (115; molar ratio 0.25) were fully nitrated and, hence, are free. Two residues (50 and 91) were partially nitrated (approximately 50%) and, thus, are assumed to be in an intermediate state. Three residues (65, 74, and 142) and one substitution (110) totaling 3.5 residues were found to be buried (or strongly H bonded) because no peptides with these tyrosine residues which had been modified could be isolated.

The Probable Role of the Buried Tyrosine Residues. A study of the CD of α_1-acid glycoprotein was carried out over a pH range from 4 to 12 (Figure 3). This property was found to change very little between pH 4 and 10 and only small changes were noted between pH 10 and 11.5. However, fundamental changes were observed between 11.5 and 12.0 which were irreversible and characterized by a shift of the negative maximum from 218 to approximately 204 nm and by the appearance of a minor positive maximum at 245 nm, suggesting the loss of a significant percentage of the secondary structure of the native protein.

The residues responsible for these conformational changes occurring during alkaline denaturation of α_1-acid glycoprotein could be identified from the changes in the CD spectra, i.e., from the pH at which half of these changes seem to have taken place. The latter suggest a pK value between 11.7 and 11.9, thus excluding the involvement of free arginine, free lysine, or free tyrosine residues. However, this pK value is in excellent agreement with the apparent pK of 11.8 of the three tightly bonded tyrosine residues reported earlier by Yamagami et al.

Table II: Amino Acid Sequences[a] of Peptides and Glycopeptides with Nitrated Tyrosine Residues.

Peptide	Sequence
G-Y1	8 Leu-Val -Pro -Val -Pro-Ile -Thr-Asn*[b]-Ala -Thr-Leu-Asp -Glu-Ile -Thr-Gly-Lys-Trp-Phe-TYR-Ile -Ala-Ser-Ala 27 E E D D D D
Y2	33 Arg -Asn -Glu -Glu-TYR 37 D D D D
G-Y3	32 Phe-Arg -Asn -Glu -Glu-TYR-Asn-Lys -Ser -Val-Glu-Glu -Ile -Gln-Ala-Thr 37 * E E E D D D D
Y4	50 Phe-TYR-Phe D D
Y5	50 TYR-Phe D
G-Y6	50 TYR-Phe -Thr -Pro-Asn -Lys-Thr -Glu -Asp-Thr-Ile -Phe-Leu D D D D
G-Y7	75* Asn-Thr -Thr -TYR-Leu 98 D,E E
G-Y8	75* Asn-Thr -Ser -TYR-Leu-Asn -Val-Gln -Arg -Glu 78 E D D
G-Y9	75* Asn-Ser -Ser -TYR-Leu-Asn -Val-Gln -Arg -Glu-Asn-Gly -Thr-Val 78 * E E E
G-Y10	85* Asn-Gly -Thr -Ile -Ser -Arg -TYR-Glu -Gly -Gly-Gln-Glu -His-Phe-Ala-His-Leu 91 E D D D D
Y11	113 Gly-Ser -TYR-Leu 115 D D
Y12	116 Val-Asn -Asp -Glu -Lys-Asn -Trp-Gly -Leu -Ser -Val-TYR-Ala-Asp-Lys-Pro-Glu-Thr 127 E E E E D D D D
Y13	123 Gly -Leu -Ser -Val-TYR-Ala-Asp-Lys-Pro-Glu-Thr 127 D D D D
Y14	125 Ser -Phe-TYR-Ala-Asp-Lys-Pro-Glu-Thr-Thr-Lys -Glu-Glu-Leu-Gly-Glu 127 D D D D
Y15	149 Arg-Ile -Pro -Lys -Ser -Asp -Val-Val -TYR-Thr 157 D D D D

[a] The amino-terminal sequences of these peptides were established by the Edman ($\bar{E}$) or dansyl ($\bar{D}$) procedure. [b] The asterisks indicate the position of the attachment of the carbohydrate unit.

(1968). Hence, these three buried residues seem to be responsible to a large extent for the stability of the secondary structure of α_1-acid glycoprotein.

The State of the Tryptophan Residues. The Number of Free Tryptophan Residues. This value was apparently two as judged from the absorbance measurements at 410 nm of the modified protein agreeing with our earlier report that two HNB residues had been introduced (Yamagami and Schmid, 1967). However, determination by amino acid analysis indicated that only 0.4 residue was reactive (for explanation of this discrepancy see below). In control experiments the stability of the HNB–tryptophan linkage was investigated: when HNB–tryptophan was subjected to alkaline hydrolysis with 5 N NaOH, no free tryptophan was formed, agreeing with the report by Horton and Koshland (1972).

The Amino Acid Sequence of the Tryptophan-Containing Peptides and the Location of These Residues in the Amino Acid Sequences of the Protein. The amino acid compositions of the tryptophan-containing peptides (Table III) indicated that only this amino acid was modified agreeing with the report by Horton and Koshland (1972). Further, as judged by high voltage electrophoresis analysis of the alkaline hydrolysate of the modified protein, only monosubstitution of the tryptophan was obtained.

Only two peptides (H-W1, H-W2) with a modified tryptophan could be isolated from the peptic digest of the modified

α_1-acid glycoprotein. They contained the same residue (122) (Table III) suggesting that approximately one third of this tryptophan was substituted, an observation which is in agreement with the mentioned direct determination of the modified tryptophan residues by amino acid analysis. To confirm the partial reaction of residue 122, the sequences of the peptides with the same tryptophan residue which, however, had not been modified, were also established (Table IV) confirming that residue 122 was indeed only partially reactive. The remaining two tryptophan residues (25, 160) proved to be unreactive and were assumed to be buried. It should be added that the molar recovery of the peptides with the same tryptophan residues varied between 45 and 55%. Further, six peptide fractions, which exhibited a yellow color in alkaline solutions, did not possess any tryptophan judging from the negative Ehrlich reaction.[5] Moreover, these six fractions when subjected to alkaline hydrolysis and subsequent high voltage electrophoresis at pH 2, proved to be indeed devoid of modified and unmodified tryptophan.

The State of the Disulfide Bonds. The molar contents of AEL- and CM-cysteine of the four CNBr fragments prepared as described in Methods were as shown in Table V. The content of cystine modified by both procedures amounted to 2 mol for each of the first two fragments and 1 mol for each of the latter two fragments. More important, the two half-cystine residues in the latter two fragments were only partially reduced under relatively mild conditions. Therefore, the half-cystine of the other fragments must have been also only partially reduced under the same conditions. Consequently, the two disulfide bonds whose positions have been established earlier (Schmid et al., 1974) must be partially buried.

The State of the Methionine Residues. Carboxymethylation of α_1-acid glycoprotein for a period of 8 days (Figure 4) revealed that the methionine residues reacted very slowly. After 1 day only 0.25 residue and, after 8 days, 1 of the 1.25 residues was substituted. These findings show that residue 111 is in an intermediate state. In absence of further chemical investigation it is not possible to indicate which state methionine 156 (a substitution present in a molar ratio of 0.25) assumes in the native protein. In the presence of 6 M urea (Figure 4) the carboxymethylation of all methionine residues proceeded to completion within 5 min.

The State of the Histidine Residues. The Number of Free and Buried Histidine Residues. Upon carboxymethylation of α_1-acid glycoprotein for a period of 8 days apparently 2 of the 3 histidine residues (97, 100, and 171) were substituted (Figure 5). In view of the data presented below, it is better to interpret these data to mean that, during this reaction period, a total of two carboxymethyl groups were introduced into the three histidines. On ultracentrifugation and starch gel electrophoresis the carboxymethylated protein (8 days) appeared homogeneous, indicating that this reaction is mild and does not seem to denature the protein. The observed sedimentation coefficient of the modified protein was essentially the same as that of the native protein ($s_{20,w}{}^{1\%}$ 2.8S), and as expected the apparent electrophoretic mobility was slightly higher than that of the untreated protein. In the presence of 6 M urea all three histidine residues were essentially modified after a reaction time of only 3 days. The difference in the reactivity of the histidine residues was demonstrated when an even shorter reaction time was employed. After 2 days one carboxymethyl

[5] It should be noted that Sephadex, which had been used for the removal of the excess HNBB from the reaction mixture, turned yellow in alkaline solutions and that this chromophore could not be extracted at acidic or alkaline pH values nor with organic solvents at pH 2 or 10.

Table III: Amino Acid Composition of Tryptophan-Containing Peptides.[a]

AA[d]	H-W1[b]	H-W2	W3	W4	W5	W6	W7	W8	W9	W10	W11	W12	W13	W14
Asp	3.6 (4)	1.5 (2)	1.3 (1)	1.0 (1)	1.0 (1)	0.9 (1)	3.4 (4)	2.7 (3)	2.5 (3)	3.2 (3)	3.1 (3)	0.7 (1)	3.2 (3)	1.9 (2)
Thr			0.9 (1)	0.8 (1)	1.1 (1)	0.4[c]							0.7 (1)	
Ser	0.6 (1)	0.7 (1)				0.5[c]	1.1 (1)		0.6 (1)	0.7 (1)	0.9 (1)	0.7 (1)	0.8 (1)	
Glu	1.1 (1)	1.0 (1)	0.6 (1)	1.0 (1)		1.0 (1)	1.2 (1)	0.8 (1)	0.9 (1)	1.0 (1)	1.3 (1)	0.8 (1)	2.6 (3)	1.0 (1)
Pro												1.0 (1)	0.7 (1)	
Gly	1.0 (1)	1.0 (1)	0.8 (1)	0.8 (1)	1.2 (1)	1.0 (1)	1.4 (2)	0.8 (1)	0.8 (1)		1.2 (1)			
Ala													1.2 (1)	
Val	1.3 (1)						0.8 (1)		0.5 (1)	1.8 (2)			1.0 (1)	
Cys													0.5 (1)	0.1 (1)
Met													0.1 (1)	
Ile				0.7 (1)	1.0 (1)	0.6 (1)				0.9 (1)	0.7 (1)			
Leu	1.0 (1)	0.5 (1)	0.4 (1)				1.0 (1)	1.0 (1)	0.6 (1)	0.8 (1)	1.6 (2)	0.7 (1)	1.4 (1)	
Tyr													0.9 (1)	
Phe						0.5 (1)								
Trp	ND (1)	ND (1)	ND (1)	ND (1)	ND (1)	ND (1)	ND (1)	ND (1)	ND (1)	ND (1)	ND (1)	ND (1)	ND (1)	ND (1)
Lys	1.0 (1)	0.8 (1)	1.0 (1)	0.8 (1)	1.1 (1)	0.6 (1)	0.7 (1)	1.0 (1)	1.0 (1)	1.0 (1)	1.0 (1)	1.0 (1)	4.5 (5)	2.2 (2)
His													0.4 (1)	
Arg					1.0 (1)								0.8 (1)	
No. of R[e]	11	8	7	7	7	8	12	8	10	11	11	7	23	7
Yield (%)	20	1	22	2	22	1	4	8	9	10	4	4	22	25
Position in sequence	115–125	118–125	19–25	19–25	19–25	19–26	113–124	116–123	116–125	116–126	116–126	119–125	153–175	159–165

[a] Under the conditions of acid hydrolysis, tryptophan was destroyed; however, these peptides were Ehrlich positive. [b] The peptides with an HNB-substituted tryptophan residue are marked with the prefix H. [c] At position 22 there is an amino acid substitution of a serine for a threonine. [d] AA, amino acid. [e] R, residues.

Table IV: Amino Acid Sequences[a] of Peptides with Modified and Unmodified Tryptophan Residues Derived from α_1-Acid Glycoprotein.

Peptide	
	A. Peptides with Partially Reactive Tryptophan Residue
	Number 1. Peptides with a Reacted Tryptophan Residue
	122
H-W1	Asp-Val-Asn-Asp-Glu-Lys-Asn -TRP-Gly-Leu-Ser
	122
H-W2	Asp-Glu-Lys-Asn -TRP-Gly-Leu-Ser
Peptide	Number 2. Peptides with an Unreactive Tryptophan Residue
	122
W7	Gly-Ser-Asp-Val-Asn-Asp-Glu-Lys-Asn -TRP-Gly-Leu
	122
W8	Leu-Asn-Asp-Glu-Lys-Asn -TRP-Gly
	122
W9	Val-Asn-Asp-Glu-Lys-Asn -TRP-Gly-Leu-Ser
	122
W10	Val-Asn-Asp-Glu-Lys-Asn -TRP-Gly-Leu-Ser-Val
	122
W11	Leu-Asn-Asp-Glu-Lys-Asn -TRP-Gly-Leu-Ser-Val
	122
W12	Glu-Lys-Asn -TRP-Gly-Leu-Ser
	B. Peptides with Unreactive Tryptophan Residues
	25
W3	Asp-Gln-Leu-Thr-Gly-Ser -TRP
	25
W4	Asp-Gln-Ile -Thr-Gly-Ser -TRP
	25
W5	Asp-Arg-Ile -Thr-Gly-Ser -TRP
	160
W13	Ser-Asp-Val-Met-Tyr-Thr-Asp-TRP-Lys -Lys-Asp-Cys-Glu-Pro-Leu-Glu -Lys-Gln-His-Glu-Lys-Arg-Lys
	160
W14	Asp-TRP-Lys -Lys-Asp-Cys-Gln

[a] The amino-terminal sequences of most peptides were established by the dansyl procedure (underscored).

Table V: Stepwise Reduction of the Disulfide Bonds of α_1-Acid Glycoprotein.

Sulfhydryl Derivative	CNBr Fragment[a] I	II	III	IV
AE-Cys	1.2	1.1	0.7	0.5
CM-Cys	0.9	1.0	0.3	0.4

[a] Expressed in moles per mole of CNBr fragment.

Table VI: Carboxymethylation of the Histidine Residues of α_1-Acid Glycoprotein.

Period of Carboxymethylation (Days)	CNBr-I		CNBr-II	
	His[a]	3-CM-His[a]	His[a]	3-CM-His[a]
2	1.6	0.4	0.9	0.1
8	1.1	0.8	0.8	0.2

[a] Expressed in moles per mole of CNBr fragment.

group was introduced into the native protein while in presence of 6 M urea 2.5 residues were modified. When the carboxymethylated protein obtained after reaction times of 2 and 8 days was cleaved with CNBr, and CNBr-I and CNBr-II (Table VI) were analyzed for unsubstituted and substituted histidine residues, it was found that residue 171 of CNBr-II had reacted very slowly. The two histidines of CNBr-I had been substituted to a somewhat higher degree. Based on these data it is not possible to indicate if only one or both histidines of CNBr-I reacted partially. However, in an unpublished investigation (K. Schmid) using the procedure of Chou and Fasman (1974) it could be predicted that the polypeptide segment with residues 95 through 104 assumes a helix and, thus, the two histidines 97 and 100 would be located on approximately the same side of this conformation. Therefore, it may be speculated that these two residues have a comparable reactivity and, thus, both are in a very similar intermediate state.

The State of the Lysine Residues. Substitution of the ϵ-amino groups of α_1-acid glycoprotein by trinitrophenylation indicated that after 50 min approximately 10 of these 12 amino groups were modified (Figure 6). Subsequent dinitrophenylation of the Tnp-modified, but unfolded protein followed by total hydrolysis, redinitrophenylation, and isolation of bis(α,ϵ-Dnp-lysine) yielded 1.9 mol of this lysine derivative per mol of glycoprotein.

In a search for the apparently buried lysine residues, the peptides with ϵ-Dnp-lysine were isolated from a proteolytic digest of trinitrophenylated-dinitrophenylated α_1-acid glycoprotein. A large number of Dnp-peptides were obtained which differed in amino acid composition but each peptide accounted for only 0.01–0.10 mol per mol of protein. This finding suggests that all lysine residues are free but the trinitrophenylation reaction reaches a degree of completeness of about 85%. The incompleteness of this reaction should be expected from the fact that the trinitrophenylation is accompanied by the formation of an aggregated state of this modified protein. The sedimentation coefficient was found to be 8S as compared with a value of 2.8S of the native protein.

Dinitrophenylation (without preceding trinitrophenylation) of α_1-acid glycoprotein which was carried out in the presence of 66% ethanol led to the substitution of all lysine residues demonstrating that this reaction is complete within 2 h. The completeness of this reaction was further shown by the absence

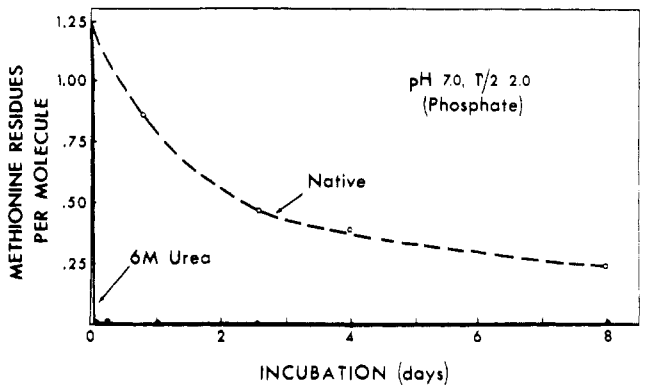

FIGURE 4: Carboxymethylation of the methionine residues of α_1-acid glycoprotein. The number of unreacted methionine residues is plotted as a function of time over an 8-day reaction period (dotted line). The reaction of the protein in the presence of 6 M urea is indicated by the solid line.

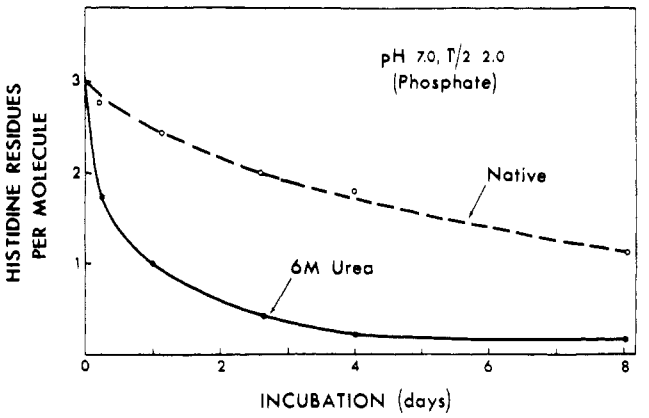

FIGURE 5: Carboxymethylation of the histidine residues of α_1-acid glycoprotein. The number of unreacted histidine residues is plotted as a function of time over an 8-day reaction period (dotted line). The reaction of the protein in the presence of 6 M urea is indicated by the solid line.

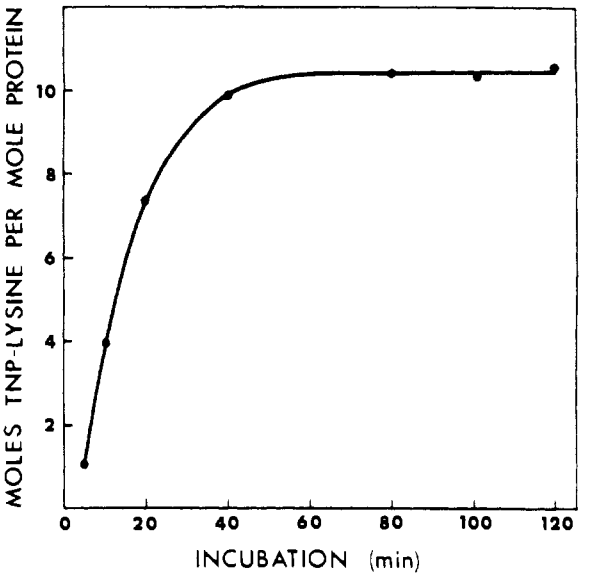

FIGURE 6: The trinitrophenylation of the lysine residues of α_1-acid glycoprotein. The number of moles of ϵ-trinitrophenyl residues introduced per mole of protein is plotted as a function of the reaction time.

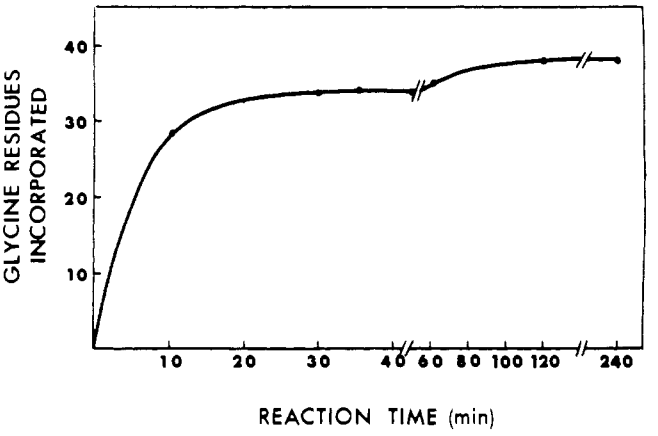

FIGURE 7: The reaction of glycine methyl ester with the carboxylic groups of α_1-acid glycoprotein. The number of moles of incorporated glycine per mole of protein is plotted as a function of reaction time. These numbers represent the total moles of glycine found minus the number of glycine residues known to be present in the untreated protein.

of free lysine in the acid hydrolysate of the dinitrophenylated protein. Under the conditions of this reaction (pH 8.0, 1.0 M $NaHCO_3$, 66% ethanol, 22 °C), the protein was denatured as judged by CD measurements.

The State of the Carboxylic Groups. The amidation of the carboxylic groups of α_1-acid glycoprotein was initially very rapid (Figure 7). After 15 min, 32 of these groups were modified and thus are free. Probably all 14 sialyl residues of this protein are included in this group, because they can be cleaved with neuraminidase. The number of amidated residues reached a maximum value after a reaction time of about 2 h and approximately 6 more carboxyl groups were substituted. These residues are probably in an intermediate state. Pertinent to this interpretation are the changes in the CD properties observed during this modification reaction (Figure 8). Small changes in the CD became apparent already after a reaction time of 10 min when approximately 25 carboxyl residues had been amidated. During the following 50 min, while 8 additional residues were glycylated, a gradual change in this property was observed involving a blue shift of the negative maximum and a concurrent decrease in the amplitude of the positive maximum. During the second hour of the reaction, although only 5 additional carboxyl groups reacted, fundamental changes in the CD properties were observed, indicating that the protein had lost a large portion of its native conformation. It should be noted that the position of the negative maximum of the denatured protein was found at approximately 204 nm as reported above for the alkaline-denatured protein. It is possible that this finding is characteristic for glycoproteins. The remaining two residues, which are assumed to be buried, reacted only after complete unfolding of the glycylated protein. When the glycine methyl ester-modified protein obtained after a reaction time of 2 h was treated with α-aminobutyric acid methyl ester in the presence of 8 M urea, 2 mol of this latter amino acid was introduced.

The present study on the topography of α_1-acid glycoprotein is of particular interest in regard to the methods employed for the determination of the exact number of the buried residues of certain amino acids of this protein. In earlier investigations one specific reagent was used to couple a free amino acid. In the present investigation, however, since the ratio of free to buried residues is very high, it was found advantageous to utilize two specific reagents, one for the determination of the free and another for the measurement of the buried residues of the same amino acid. The following examples illustrate this point. The numbers of buried lysine and carboxylic groups, which were very small, were difficult to measure as the dif-

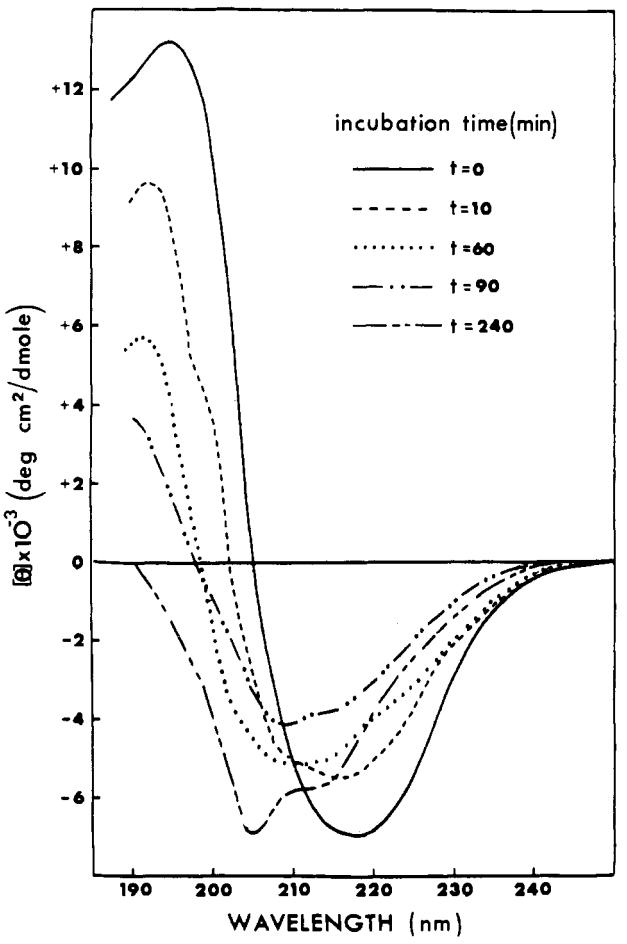

FIGURE 8: Circular dichroism of α_1-acid glycoprotein as a function of reaction time during amidation of its carboxyl groups with glycine methyl ester. Not included in this figure are the 30- and 120-min curves. The former lies almost exactly between the 10- and 60-min curves and the latter between the 90- and 240-min curves.

ferences between the total and free amino acids because of the error of the technique used. Therefore, these residues were determined directly by the following differential procedure. First, the protein was modified in the native state with a specific reagent and, secondly, in order to measure the buried residues, the modified protein was subsequently unfolded and then treated with another specific reagent. This general procedure allows precise measurements of buried amino acids even when present in very small quantities. In the case of lysine, substitution with Tnp was followed by that with Dnp and, in case of the carboxyl groups, the modification reaction with glycine methyl ester was succeeded by that of α-aminobutyric acid methyl ester. As to the cystine, the substitution with ethyleneamine was followed with that of iodoacetate.

For the correct interpretation of the results obtained, it should be emphasized that additional chemical investigations (amino acid composition, etc.) of the peptides with the buried residues are essential because quantitation of the substituted, specific reagent alone may in certain cases be totally misleading. For example, chemical analysis of the isolated Dnp-peptides revealed that the two apparently buried lysine residues proved to be derived from a large number of lysine peptides, each present in a small molar fraction due to the incompleteness of the Tnp reaction. These problems were resolved in the procedure employed for the determination of the free and buried tryptophan and tyrosine by isolating and sequencing the peptides with the reactive and unreactive residues.

In the present study we have determined the state of all carboxylic groups, half-cystines, histidines, methionines, lysines, tryptophans, and tyrosines of α_1-acid glycoprotein. Thus, the state of almost half of the amino acid residues of this glycoprotein has been established. Based on these data it should be possible to arrange spatially the conformations of α_1-acid glycoprotein so that the free residues are pointing toward the solvent whereas most buried residues and a large number of the other hydrophobic residues would be located in the interior of the protein. Thus, based on these data and the conformational regions calculated from the established amino acid sequence of α_1-acid glycoprotein, it should be possible to build a reasonable three-dimensional model of this human plasma glycoprotein.

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References

Barman, T. E., and Koshland, Jr., D. E. (1967), *J. Biol. Chem. 242,* 5771–5776.

Bürgi, W., and Schmid, K. (1961), *J. Biol. Chem. 236,* 1066–1074.

Castellino, F. J., and Hill, R. L. (1970), *J. Biol. Chem. 245,* 417–424.

Chou, P. Y., and Fasman, G. D. (1974), *Biochemistry 13,* 211–222.

Cohn, E. J., Strong, L. E., Hughes, W. L., Jr., Mulford, D. J., Ashworth, J. N., Melin, M., and Taylor, H. L. (1946), *J. Am. Chem. Soc. 68,* 459–475.

Cole, R. D. (1967), *Methods Enzymol. 11,* 315–317.

Frankel-Conrat, H., Harris, J. T., and Levy, A. L. (1955), *Methods Biochem. Anal. 2,* 359–425.

Gray, W. R. (1972), *Methods Enzymol. 25,* 121–138.

Gurd, F. R. N. (1972), *Methods Enzymol. 25,* 424–438.

Hoare, D. G., and Koshland, D. E., Jr. (1967), *J. Biol. Chem. 242,* 2447–2453.

Horton, H. R., and Koshland, D. E., Jr. (1972), *Methods Enzymol. 25,* 468–482.

Hugli, T. E., and Moore, S. (1972), *J. Biol. Chem. 247,* 2828–2834.

Ikenaka, T., Bammerlin, H., Kaufmann, H., and Schmid, K. (1966), *J. Biol. Chem. 241,* 5560–5563.

Ikenaka, T., Ishiguro, M., Emura, J., Kaufmann, H., Isemura, S., Bauer, W., and Schmid, K. (1972), *Biochemistry 11,* 3817–3829.

Ishiguro, M., Yamamoto, A., and Schmid, K. (1969), *Biochim. Biophys. Acta 194,* 91–101.

Jeanloz, R. W. (1972) in Glycoproteins, Gottschalk, A., Ed., New York, N.Y., Elsevier, pp 565–611.

Nigen, A. M., and Gurd, F. R. N. (1973), *J. Biol. Chem. 248,* 3708–3715.

Nimberg, R., Motoyama, T., and Schmid, K. (1971), *J. Biol. Chem. 246,* 5817–5821.

Raftery, M. A., and Cole, R. D. (1966), *J. Biol. Chem. 241,* 3457–3461.

Riordan, J. F., Wacker, W. E. C., and Vallee, B. L. (1965), *Biochemistry 4,* 1758–1765.

Riordan, J. F., Sokolovsky, M., and Vallee, B. L. (1967), *Biochemistry 6,* 358-361.
Sairam, M. R., Papkoff, H., and Li, C. H. (1972), *Biochem. Biophys. Res. Commun. 48,* 530-537.
Sanger, F. (1949), *Biochem. J. 45,* 563-574.
Sanger, F., and Thompson, E. O. P. (1963), *Biochim. Biophys. Acta 71,* 468-471.
Satake, K., Okuyama, T., Ohashi, M., and Shinoda, T. (1960), *J. Biochem. (Tokyo) 47,* 654-660.
Schmid, K. (1953), *J. Am. Chem. Soc. 75,* 60-68.
Schmid, K. (1976), in The Plasma Proteins, 2nd ed, Putnam, F. W., Ed., New York, N.Y., Academic Press.
Schmid, K., Bürgi, W., Collins, J. H., and Nanno, S. (1974), *Biochemistry 13,* 2694-2697.
Schmid, K., Kaufmann, H., Isemura, S., Bauer, F., Emura, J., Motoyama, T., Ishiguro, M., and Nanno, S. (1973), *Biochemistry 12,* 2711-2724.
Sokolovsky, M., Fuchs, M., and Riordan, J. F. (1970), *FEBS Lett. 7,* 167-170.
Spiro, R. G. (1966), *Methods Enzymol. 8,* 3-26.
Woods, K. R., and Wang, K.-T. (1967), *Biochim. Biophys. Acta 133,* 369-370.
Yamagami, K., Labat, J., Pandey, R. S., and Schmid, K. (1968), *Biochemistry 7,* 2873-2879.
Yamagami, K., and Schmid, K. (1967), *J. Biol. Chem. 242,* 4176-4181.

Structure–Function Relationships of Scorpion Neurotoxins†

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ABSTRACT: Chemical modification of some trifunctional amino acid residues in toxins I, II, and III of the scorpion *Androctonus australis* Hector have been performed. The results indicate: (1) Reduction and methylation of one disulfide bridge destroy toxic activity of toxin II. (2) The only tryptophan residue of toxin II (position 38) is not included in the active site of the molecule. (3) Modification of five carboxylates out of the seven contained in toxin II suppresses the toxic activity. (4) Acetylation of the lysine and tyrosine residues in toxin II leads to the loss of both toxic and antigenic activity. Treatment of the acetylated toxin by hydroxylamine restores partially the antigenic activity. In the case of toxin I, total acetylation abolishes only the toxic activity. It is concluded that at least one tyrosine residue must be involved in an antigenic site of toxin II. (5) Citraconylation of toxins II and III leads to complete loss of toxicity; decitraconylation restores full activity. (6) Guanidination of toxin II does not affect its toxicity significantly. (7) Alkylation of toxin II by iodoacetic acid affects both amino groups and histidine residues. The loss of toxicity is mainly due to the modification of the lysine residues. In the case of toxin I, the kinetics of toxicity loss closely parallel the covalent modification of one lysine residue.

Scorpion neurotoxins are made of single peptide chains of about 60 amino acid residues cross-linked by four disulfide bridges. The complete sequences of 7 neurotoxins and parts of the sequences of 14 other toxins have been established (Rochat, H., et al., 1970a, 1972, 1974a; Babin et al., 1974, 1975; Zlotkin et al., 1973). From the comparison of the N-terminal sequences, it was shown that they form a family of homologous proteins (Rochat, H., et al., 1970b, 1974a; Babin et al., 1975). The positions of the disulfide bridges in toxin II of *Androctonus australis* Hector have been determined (Kopeyan et al., 1974). They were found to link half-cystine residue numbers 12 and 63, 16 and 36, 22 and 46, and 26 and 48. In toxin I of *Androctonus australis* Hector, two disulfide bridges were located in identical positions (Rochat, H., et al., 1970c), suggesting that the positions of the disulfide bridges may be the same in all scorpion neurotoxins.

Some physicochemical properties of toxin II of *Androctonus australis* Hector have been studied by optical rotatory dispersion and ultraviolet difference spectrophotometry (Chicheportiche and Lazdunski, 1970). Depending upon pH and temperature, the protein takes at least four different molecular forms. The form which is predominant between pH 4 and 9 is very stable under heat denaturation and treatment with 9.5 M urea.

The relationships between iodinated tyrosine residues and toxic activity have been demonstrated for both toxins I and II in the course of experiments to obtain biologically active radioiodinated toxins (Rochat, C., et al., 1972; Rochat, H., et al., 1974b).

For this paper other modifications to trifunctional amino acids have been made, and the effects of these modifications on toxic activity have been studied (cf. also Sampieri and Habersetzer-Rochat, 1974). Some further conclusions about the relationships of structure and activity in scorpion neurotoxins have been drawn.

Materials and Methods

Three neurotoxins, I, II, and III, were purified from the venom of *Androctonus australis* Hector (Rochat, C., et al., 1967; Miranda et al., 1970). These neurotoxins have, respectively, molecular weights of 6808 (63 amino acid residues), 7249 (64 amino acid residues), and 6826 (64 amino acid residues), and contain, respectively, 6, 5, and 6 lysine residues and 1, 2, and 1 histidine residues. Only one tryptophan residue is present in each of the three molecules.

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