Acknowledgments

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The Disulfide Bonds of α_1-Acid Glycoprotein†

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ABSTRACT: For the elucidation of the positions of the disulfide bonds of human plasma α_1-acid glycoprotein, the amino acid sequences of the cystine-containing peptides isolated from an enzymatic digest of this globulin were determined and compared with the earlier established, linear amino acid sequence of the protein. For this purpose the protein was first digested with pepsin at pH 2.0 and then with trypsin and chymotrypsin at pH 6.5. The resulting digest was fractionated by gel filtration through Sephadex G-25 and the obtained glycopeptide fraction was further resolved by passage through a Sephadex G-75 column. The cystine-containing glycopeptides and peptides which were then isolated in homogeneous form by chromatography on Dowex 50W-X2 were subsequently oxidized with performic acid. The two cysteic acid containing peptides resulting from each of the cystine peptides were separated from each other by the same ion exchange chromatographic procedure and their amino acid compositions and amino acid sequences were determined. The obtained data indicated that α_1-acid glycoprotein possesses two disulfide bonds and that one of these disulfide bonds links residue 5 to residue 147, while the other connects residue 72 with residue 164 of the protein.

Recently, the linear amino acid sequence of human plasma α_1-acid glycoprotein was elucidated and found to consist of 181 residues and to contain four half-cystine residues (Schmid *et al.*, 1973; Ikenaka *et al.*, 1972). In the present report we wish to describe the location of the two disulfide bonds of this protein.

Materials and Methods

α_1-Acid Glycoprotein. This globulin (for review, see Jeanloz, 1972) was isolated from Cohn fraction VI of pooled normal human plasma by a procedure described earlier (Bürgi and Schmid, 1971) and its apparent homogeneity was established by several criteria of purity (Jeanloz, 1972; Bürgi and Schmid, 1971; Ikenaka *et al.*, 1966).

Enzymatic Digestions. The peptic (a), tryptic (b), and chymotryptic (c) digestions of the protein were performed under the conditions similar to those described by Spackman *et al.* (1960): (a) pH 2.0, 24°, protein concentration 1%, enzyme–protein ratio 1:500 and 17 hr, (b) pH 6.5, 24°, protein concentration 1.3%, enzyme–protein ratio 1:250 and 4 hr, and

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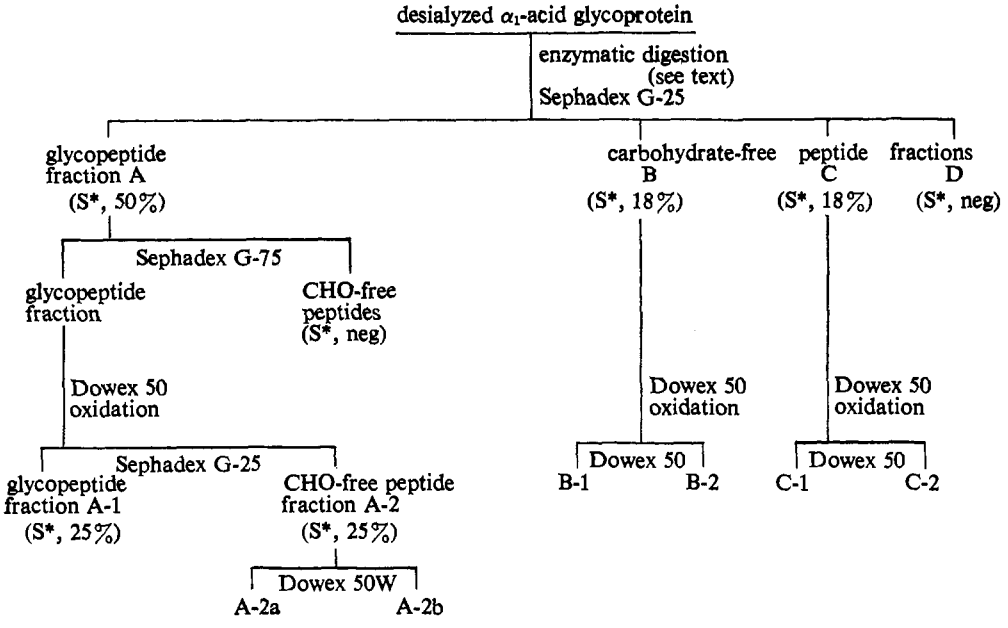

FIGURE 1: The isolation of cysteic acid containing CHO-free peptides and glycopeptides from α_1-acid glycoprotein. The following abbreviations were used: oxidation, performic acid oxidation; S*, cystine or cysteic acid content, and neg, negligible.

(c) pH 6.5, 24°, protein concentration 1.3%, enzyme–protein ratio 1:200 and 4 hr. The pH of 6.5 was kept constant by addition of 0.05 N NaOH employing a Radiometer pH-Stat (Model TTTlc). Pepsin (recrystallized), trypsin–L-1-tosylamido-2-phenylethyl chloromethyl ketone (recrystallized), and chymotrypsin (CDI) were purchased from Worthington Biochemical Corp., Freehold, N. J.

Performic Acid Oxidation. This procedure was carried out according to Hirs (1967) and Phelan and Hirs (1970).

Analytical Techniques. The effluents of the chromatographic columns were monitored by absorbancy measurements at 280 nm and in some instances by the Folin–Lowry procedure (Lowry *et al.*, 1951). The disulfide content of the effluent was estimated by the procedure of Zahler and Cleland (1968), whereas the disulfide peptides on the electrophoretograms were detected by the cystine stain of Maeda and Meienhoffer (1970). The amino acid compositions of the cysteic acid containing peptides were determined using 24-hr hydrolysates (constant boiling HCl, N_2, evacuated and sealed tubes) by a Jeol amino acid analyzer (Model 6AH), and the amino acid sequences of these compounds were established with the aid of the direct (Iwanaga *et al.*, 1967) and the indirect (Elzinga *et al.*, 1968) Edman procedures. Thin-layer plates (silica gel FG, 250 μ, purchased from Analtech Inc., Newark, Del.) were used for the identification of the phenylthiohydantoin amino acids (Needleman, 1970). High-voltage electrophoreses using Whatman No. 1 paper were carried out at 4000 V for 1.5 hr in pH 3.7 pyridine–acetic acid–water buffer (1:10:289 v/v). Further high-voltage electrophoreses were performed in 1% formic acid (pH 1.8) and in pH 9.2, 0.05 M sodium borate buffers. The technical details of many additional procedures necessary for the present study were referred to in a previous investigation (Schmid *et al.*, 1973; Ikenaka *et al.*, 1972).

Results and Discussion

In order to isolate relatively large cystine-containing peptides and glycopeptides and in order to minimize nonspecific enzymatic cleavage, α_1-acid glycoprotein was digested with three, relatively specific proteases employing low enzyme–substrate ratios and in part relatively short digestion periods. To minimize disulfide interchange these and all subsequent reactions were carried out at pH values of 6.5 or below. α_1-Acid glycoprotein (1.1 g or approximately 25 μmol) which had been desialyzed by mild acid hydrolysis without cleavage of any peptide bonds (Schmid *et al.*, 1967) was first digested with pepsin. After lyophilization the digest was dissolved in 85 ml of water, adjusted to pH 6.5 with distilled pyridine, and further digested with trypsin. Without intermediate freeze-drying, chymotrypsin was then added to the tryptic hydrolysate. The lyophilized hydrolysate which was dissolved in 20 ml of 5% acetic acid was chromatographed subsequently on an appropriate Sephadex G-25 column previously equilibrated with the same solvent and a glycopeptide and three carbohydrate-free fractions were obtained (Figure 1).

The glycopeptide fraction was further purified by gel filtration through Sephadex G-75 in 5% acetic acid, followed by chromatography on a Dowex 50W-X2 column using pyridine acetate buffer (pH 3.1) (Schroeder, 1972). The obtained cystine-containing glycopeptide was oxidized and then fractionated as indicated in Figure 1.

The carbohydrate-free, cystine-containing peptide fractions (B and C) were chromatographed on Dowex 50W-X2 columns and the resulting cystine peptides oxidized. The formed cysteic acid peptides were separated from each other by chromatography on Dowex 50W-X2 (Figure 1). It should be pointed out that only those cystine-containing peptides and glycopeptides which appeared homogeneous on high-voltage electrophoresis at three pH values are indicated in Figure 1.

The amino acid compositions of the homogeneous peptides are listed in Table I. It should also be noted that the sum of the yields of the peptides that were derived from the same sequence of the original protein are relatively high. The amino acid sequences of these peptides are listed in Table II. Peptide A-2b whose sequence was not determined is most probably identical with peptide A-2a except that the former possesses at its amino-terminus an additional lysine residue as judged by a comparison of its amino acid composition with that of peptide A-2a and the total amino acid sequence of the protein. The elucidated amino acid sequences were then compared with the earlier established linear amino acid sequence of the protein (Schmid *et al.*, 1973) in order to locate their positions

TABLE I: Amino Acid Compositions of Cysteic Acid Containing Peptides Derived from α_1-Acid Glycoprotein.

Amino Acid	Peptides (Expressed in Moles of Amino Acids per Mole of Peptide)						
	A-1	A-2a	A-2b	B-1	B-2	C-1	C-2
$CySO_3H$	0.74 (1)	0.91 (1)	0.84 (1)	0.84 (1)	0.91 (1)	0.84 (1)	0.93 (1)
Asp	2.04 (2)	0.93 (1)	1.03 (1)	1.04 (1)	0.94 (1)	0.97 (1)	1.04 (1)
Glu	2.12 (2)	2.14 (2)	2.03 (2)				
Ala				1.21 (1)		1.03 (1)	
Thr	2.30 (3)						
Leu	1.00 (1)	1.02 (1)	1.00 (1)	1.00 (1)	1.00 (1)	1.10 (1)	1.13 (1)
Ile	0.40[a]				0.98 (1)		
Pro		1.21 (1)	1.31 (1)		1.04 (1)		
Phe	0.52[a]						
Tyr	1.91 (2)						
Lys		1.91 (2)	2.92 (3)		1.04 (1)		
Arg	0.93 (1)				1.03 (1)		1.01 (1)
No. of residues	13	8	9	4	7	4	4
Yield (%)	25	40	9	18	11	23	18
Position in sequence	67–79	162–169	161–169	5–8	146–152	5–8	146–149

[a] Both isoleucine and phenylalanine are present in position 73 in α_1-acid glycoprotein *from pooled normal human plasma* (Schmid *et al.*, 1973).

TABLE II: Amino Acid Sequences of Cysteic Acid Containing Peptides Derived from α_1-Acid Glycoprotein.

Peptide	Sequence	Position of Half-cystine Residue within the Amino Acid Sequence of the Protein
A-1	Thr-Arg-Glx-Asx-Glx-$CySO_3H$[a]-$\begin{matrix}\text{Ile}\\\text{Phe}\end{matrix}$-Tyr-Asn[b]-Thr-Thr-Tyr-Leu	72
A-2a	Lys-Asp-$CySO_3$-H-Glx-Pro-Leu-Glu-Lys	164
B-1, C-1	$CySO_3H$-Ala-Asn-Leu	5
B-2	Asp-$CySO_3H$-Leu-Arg-Ile-Pro-Arg	147
C-2	Asp-$CySO_3H$-Leu-Arg	147

[a] The residue cysteic acid is abbreviated as $CySO_3H$. [b] This Asn residue carries a carbohydrate unit. For the determination of the sequence of glycopeptide A-1 and peptide A-2a the indirect Edman procedure was employed, while for the elucidation of the structures of the other peptides the direct Edman method was used.

in the structure of the original protein. This comparison confirmed that α_1-acid glycoprotein possesses two disulfide bonds and indicated that the half-cystine residue 5 is linked to half-cystine residues 147 and half-cystine residue 72 is linked to the half-cystine residue 164 of this α-globulin.

A schematic drawing of α_1-acid glycoprotein with the exact location of the two disulfide bonds is presented in Figure 2.

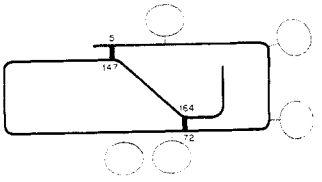

FIGURE 2: A schematic representation of α_1-acid glycoprotein with its two disulfide bonds. The single polypeptide chain of the protein is represented by the long line and the two disulfide linkages by the two short bars. The five carbohydrate units of this α-globulin are indicated by the five shaded areas which are linked to the asparagine residues 15, 38, 54, 75, and 86 of the protein.

The positions of these two cystine residues and those of the methionine residues (111 and 156[1]) explain why on CNBr cleavage of the native glycoprotein the resulting four fragments (Schmid *et al.*, 1973; Ikenaka *et al.*, 1972) are held together by the two disulfide bonds.

With the determination of the location of the two disulfides, the total amino acid sequence of α_1-acid glycoprotein, one of the most highly conjugated plasma glycoproteins studied so far, has been established.

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[1] Position 156 of α_1-acid glycoprotein from *pooled* normal human plasma contained both methionine and valine in a molar ratio of 1 : 3 (Schmid *et al.*, 1973).

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Troponin, Tropomyosin, and Actin Interactions in the Ca^{2+} Regulation of Muscle Contraction†

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ABSTRACT: The effect of Ca^{2+} on the interaction of troponin (TN) and its components with F-actin, tropomyosin, and the F-actin–tropomyosin complex has been studied. The Ca-binding component of TN (TN-C) did not bind to F-actin, F-actin–tropomyosin, or tropomyosin regardless of whether Ca^{2+} was present. A complex of TN-C and of the inhibitory component (TN-I) bound to F-actin only in the presence of tropomyosin and in the absence of Ca^{2+}. TN-I was weakly bound to F-actin and more strongly to F-actin–tropomyosin. Unfractionated troponin bound tightly to tropomyosin and to F-actin–tropomyosin but only weakly to F-actin, and in both cases the binding was not affected by Ca^{2+}. The tropomyosin-binding component of TN (TN-T) and a complex of TN-C and TN-T bound to tropomyosin and to F-actin–tropomyosin but not to F-actin alone. On the basis of these binding studies and the known positions of tropomyosin on the thin actin-containing filaments during contraction and relaxation, a model is proposed which may explain how troponin and Ca^{2+} regulate muscle contraction.

Troponin, a complex of three subunits (Greaser and Gergely, 1971; Greaser *et al.*, 1972), and tropomyosin are required to confer Ca^{2+} sensitivity on the actin–myosin interaction. Ebashi *et al.* (1969) showed that TN[1] and TM were located in the thin actin-containing filaments with TN spaced at intervals of 40 nm along the thin filament. From this spacing they estimated that TN, TM, and actin are present in a molar ratio of 1:1:7. In good agreement with this stoichiometry, Bremel and Weber (1972) have shown that one TN and one TM can block the combination of myosin with approximately seven actins. More recently Potter (1974) has shown by direct measurement that TN, TM, and actin are present in a 1:1:7 molar ratio in myofibrils and that in each TN the molar ratio of TN-T:TN-I:TN-C is 1:1:1. Hanson and Lowy (1963) originally suggested that TM may be located in the two long pitch grooves of the F-actin double helix. Recently several groups (Hanson *et al.*, 1972; Spudich *et al.*, 1972; Haselgrove, 1972; Huxley, 1972; Parry and Squire, 1973) have confirmed this suggestion on the basis of X-ray diffraction data from living muscle and three-dimensional reconstructions from electron micrographs of TN- and TM-containing actin paracrystals. It has been further deduced that TM is localized asymmetrically in the grooves between the two long pitch actin helices of the thin filaments, so that one strand of TM is in close association with one helical set of actin monomers and another TM strand with the other actin helix. In the relaxed state tropomyosin molecules are thought to sterically block the combination of myosin heads with actin. In the activated state, *viz.*, upon combination of Ca^{2+} with TN-C, TM moves into a position closer to the groove and no longer blocks the interaction of myosin.

Hitchcock and Szent-Györgyi (1973) reported that Ca^{2+} inhibited the combination of a TN-T-deficient TN (*i.e.*, a com-

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[1] Abbreviations used are: TM, tropomyosin; TN, unfractionated troponin; TN-T, tropomyosin-binding subunit of troponin; TN-I, ATPase inhibitory subunit; TN-C, Ca^{2+}-binding subunit; CT, the complex of TN-C and TN-T; CI, the complex of TN-C and TN-I; EGTA, ethylene glycol bis(β-aminoethyl ester); EDTA, ethylenediaminetetraacetic acid.