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Purification and Chemical Characterization of Salt-Extractable Glycoproteins from Porcine Mitral Valve†

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ABSTRACT: Four major glycoproteins were extracted by dilute salt solution from porcine mitral valvular tissue. Two of these major glycoproteins, porcine valve glycoprotein I and porcine valve glycoprotein III, were isolated and purified by fractionation of salt extract with ammonium sulfate followed by column chromatography on DEAE-cellulose. The purified glycoproteins appeared to be homogeneous by polyacrylamide disc electrophoresis in several buffer systems, and by Sephadex filtration. The porcine valve glycoprotein I has a molecular weight of approximately 120 000. Isoelectric focusing yielded a single band, pI = 5.8. The glycoprotein contained large amounts of acidic amino acids, and amide nitrogen. The carbohydrate moiety was composed of fucose, mannose, galactose, glucose, glucosamine, and galactosamine in the molar ratio of 5:10:15:12:7:2 per mole of glycoprotein. The second major glycoprotein, porcine valve glycoprotein III, has an approximate molecular weight of 72 000. This glycoprotein gives two bands upon analytical isoelectric focusing with isoelectric points of pI = 4.1 and 4.3. Porcine valve glycoprotein III contained large amounts of acidic amino acids and low amounts of amide nitrogen. Its carbohydrate moiety was composed of glucose, galactose, mannose, fucose, glucosamine, and sialic acid in the ratio of 3:3:2:1:4:1 mol/mole of glycoprotein. This glycoprotein was similar to a glycoprotein preparation isolated from porcine aortic intima by P. V. Wagh and B. I. Roberts (1972), *Biochemistry 11,* 4222.

Mesenchymal glycoproteins are considered as major components of vascular tissue. The isolation of glycoproteins from vascular tissue was achieved by Robert and his co-workers (Robert et al., 1965; Moczar and Robert, 1970) using trichloroacetic acid to solubilize tissue collagen, followed by extraction with urea. Barnes and Partridge (1968) utilized cold alkali to extract glycoproteins from human thoracic aorta. A simple salt extraction procedure for the isolation and purification of glycoproteins from bovine and porcine aortas was reported by Radhakrishnamurthy et al. (1964, 1966) and by Wagh and Roberts (1972). This gentle extraction procedure has the advantage of solubilizing glycoproteins with the least alteration from their native form.

The biological importance of mesenchymal glycoproteins was recently emphasized by Goldstein and his co-workers (Goldstein et al., 1968) and by Kawakita and Iwamoto (1975) who reported that an antigenic cross-reactivity existed between

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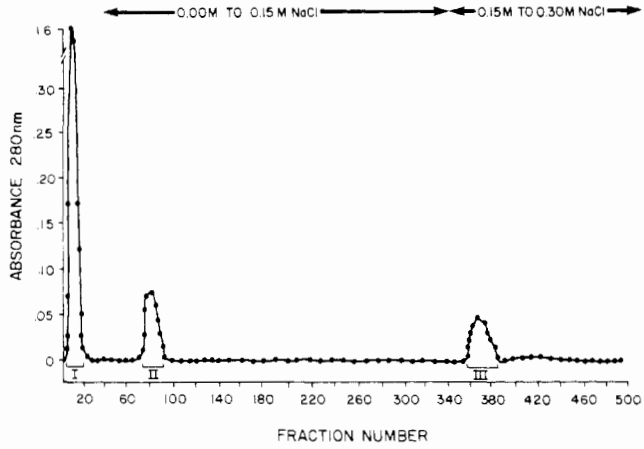

FIGURE 1: DEAE-cellulose column chromatography of 60–90% ammonium sulfate saturated fraction. DEAE-cellulose (Schleicher and Schuell, Selectacel type 40, capacity 0.9 meq/g) was equilibrated with Tris–EDTA buffer. Column size was 2.5 × 40 cm. The elution was initiated with 400 ml of Tris–EDTA buffer. The first linear gradient was from 0 to 0.15 M NaCl in equilibrating buffer (1500 ml each). The second linear gradient set up was from 0.15 M NaCl to 0.3 M NaCl in equilibrating buffer (800 ml each). Ten-milliliter fractions were collected and assayed for OD 280 nm.

the streptococcal cell-wall carbohydrate and the structural glycoproteins of mitral valve tissue. This finding and the utilization of porcine mitral valves for replacement of diseased valves in human prompted us to study glycoprotein components of porcine mitral valves. The present report describes the extraction of several glycoproteins from porcine valvular tissue using dilute salt solution, and the chemical characterization of two major glycoproteins.

Materials and Methods

Extraction of Glycoproteins. All procedures were performed at 4 °C, unless otherwise mentioned. Porcine mitral valves were obtained from a local slaughterhouse and transported to the laboratory in ice-cold saline. They were cleaned of adventitia and washed several times with saline, frozen on a block of dry ice, and chopped to small pieces. Minced valve tissue was then extracted three times over a period of 3 days with 20 volumes of extraction buffer (0.1 M phosphate, pH 7.0, containing 0.3 M NaCl) with continuous stirring. The combined extract was centrifuged for 1 h at 8000 rpm and the supernatant passed through a column (2.5 × 4 cm) of glass wool to remove small fragments of tissue not precipitated by centrifugation.

Ammonium Sulfate Precipitation. Solid ammonium sulfate was added to the combined salt extract to 40% saturation. The solution was stirred overnight at 4 °C and centrifuged for 1 h at 10 000 rpm to remove the precipitated material. Ammonium sulfate was added to the supernatant to 60% saturation, the mixture stirred overnight, and the precipitate collected as before. Finally the saturation of ammonium sulfate in the supernatant was brought to 90% and the 60–90% ammonium sulfate precipitate recovered as described above. This precipitate was dissolved in about 50 ml of 0.005 M Tris-HCl buffer, pH 7.0, containing 0.001 M EDTA (Tris–EDTA buffer), and dialyzed against the same buffer. The small amount of precipitate formed during dialysis was removed by centrifugation and discarded.

Ion-Exchange Column Chromatography. The elution profile of the 60–90% ammonium sulfate fraction from DEAE-cellulose is shown in Figure 1. Fractions constituting peak I (material not exchanged by DEAE), peak II (material eluted at 0.01–0.02 M NaCl), and peak III (material eluted at 0.16–0.19 M NaCl) were pooled separately, dialyzed against distilled water, and lyophilized.

Sephadex Column Chromatography. Columns, 1 × 85 cm and 1.5 × 52 cm of Sephadex G-100 and Sephadex G-150, respectively, were equilibrated with 0.01 M phosphate buffer, pH 7.4. Columns were calibrated by eluting proteins and glycoproteins of known molecular weights.

Polyacrylamide Disc Electrophoresis. Discontinuous (Davis, 1964) and continuous (Peacock et al., 1965) analytical polyacrylamide disc electrophoresis was performed on the various fractions and on the purified glycoproteins. Disc electrophoresis, in the presence of sodium dodecyl sulfate for determination of molecular weight, was also performed according to the method of Weber and Osborn (1969).

A sample containing 30–60 μg of protein was subjected to polyacrylamide disc electrophoresis. Gels were stained with amido black to visualize proteins and with periodic acid–Schiff reagent to visualize glycoproteins (Zaccharias et al., 1969).

Analytical Isoelectric Focusing. Purified glycoprotein samples were subjected to analytical thin-layer isoelectric focusing in ampholine PAG plates (LKB Produkter, Stockholm, Sweden), pH range 3.5–9.5.

Analysis of Amino Acids and Amino Sugars. For amino acid analysis, samples were hydrolyzed in 6 N HCl under nitrogen in sealed glass ampules at 110 °C for 28, 36, 48, and 72 h. Hydrolyzed samples were evaporated to dryness, redissolved in 0.1 N HCl, and analyzed. Half-cystine contents were determined independently as cysteic acid (Moore, 1963). Tryptophan was estimated spectrophotometrically (Goodwin and Morton, 1946).

Amino sugars were analyzed on the amino acid analyzer, after hydrolysis of samples with 4 N HCl for 4 h at 105 °C, and removal of HCl by the use of the Bio-dryer (Virtis Research Equipment, Model 10-310). Hexosamine contents of the hydrolyzed samples were also determined by modification of the Elson–Morgan method (Elson and Morgan, 1933; Rondle and Morgan, 1955) with glucosamine as standard. The amino sugar analysis of the glycoproteins by the two methods gave similar results.

Gas Chromatography of Neutral Sugars. For analysis of neutral sugar components, samples were prepared according to the procedure of Lehnhardt and Winzler (1968). Analysis was performed using a Hewlett-Packard Model 402 FM gas chromatograph with a 1.83 m × 2 mm (i.d.) glass U-shaped column packed with 3% OV 225 on 80–100 mesh Supelco port (Supelco, Inc., Bellefonte, Pa. 16823). Column temperature was maintained at 205 °C, injection port and detection port were 225 °C, and carrier helium flow was 40 ml/min. This system resolved fucose, mannose, galactose, and glucose completely.

Other Analytical Techniques. Sample solutions (1–2 mg/ml in water) suspected of being contaminated with glycolipids were extracted with 10 volumes of chloroform:methanol 2:1 (v/v) for 2 h at room temperature, as described by Folch et al. (1957). The chloroform:methanol and aqueous layers were separated carefully, evaporated to dryness, and analyzed for carbohydrate and protein contents. *Protein* contents of the samples were determined by the method described by Campbell and Sargent (1967) with bovine serum albumin as standard. *Amide nitrogen* content of the desialized and hydrolyzed

[1] Abbreviations used are: DEAE, diethylaminoethyl; EDTA, (ethylenedinitrilo)tetraacetic acid; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; i.d., inside diameter; OD, optical density.

TABLE I: Percent Distribution of Sialic Acid, Neutral Sugars, and Proteins in Various Ammonium Sulfate Saturated Fractions.

	Weight[a] (mg)	Sialic Acid (%)	Neutral Sugars (%)	Protein (%)
0.3 M salt extract[b]	139.00	100 (4.0 mg)	100 (9.0 mg)	100 (126 mg)
0–40% ammonium sulfate saturated fraction	71.40	35.0	36.0	53.0
40–60% ammonium sulfate saturated fraction	3.13	4.0	5.0	2.0
60–90% ammonium sulfate saturated fraction	64.40	61.0	59.0	45.0

[a] Approximately 100 g (wet weight) of porcine mitral valves were extracted. For details see text. [b] This value was calculated by adding sialic acid, neutral sugars, and protein contents.

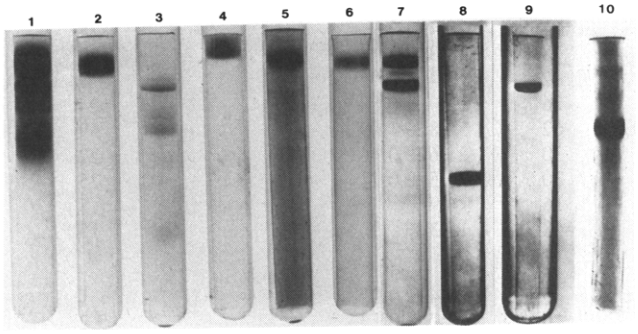

FIGURE 2: Polyacrylamide disc electrophoresis, from left to right: (1) fraction I from DEAE-cellulose column in buffer system of Davis; (2) purified valve glycoprotein I in buffer system of Davis; (3) purified valve glycoprotein I in buffer system of Weber and Osborn; (4) purified valve glycoprotein I in buffer system of Weber and Osborn in absence of 2-mercaptoethanol; (5) purified valve glycoprotein I in the buffer system of Davis with 0.1% sodium dodecyl sulfate; (6) purified valve glycoprotein I in buffer system of Peacock et al.; (7) fraction II from DEAE-cellulose column in buffer system of Davis; (8) purified valve glycoprotein III in buffer system of Davis; (9) purified valve glycoprotein III in buffer system of Weber and Osborn; and (10) purified valve glycoprotein III in buffer system of Peacock et al. Migration is downward toward anode.

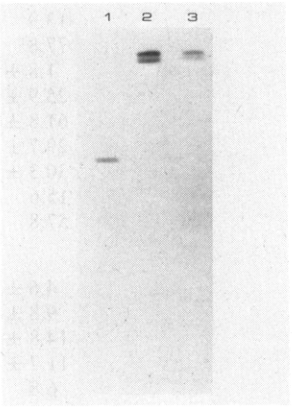

FIGURE 3: Analytical isoelectric focusing, from left to right: (1) porcine valve glycoprotein I (1 mg/ml), (2) porcine intimal glycoprotein (2 mg/ml), and (3) porcine valve glycoprotein III (1 mg/ml). Migration is upward toward anode.

samples was determined according to a procedure described by Spiro and Spiro (1962). Total *hexose* content was estimated by the phenol–sulfuric acid method with galactose as standard (Dubois et al., 1965), *sialic acid* content by the thiobarbituric acid assay procedure of Aminoff (1961) with *N*-acetylneuraminic acid as standard, *uronic acid* content by the carbazole method according to Bitter and Muir (1962) with glucuronic acid as standard, and *hydroxyproline* content by the method of Neuman and Logan (1950). *Sulfate* content was measured by the method of Antonopoulos (1962).

Results

Table I summarizes the distribution of proteins, neutral sugars, and sialic acid in fractions obtained at various levels of saturation with ammonium sulfate. These data show that the major portion of the sialic acid containing material was recovered in the 60–90% ammonium sulfate precipitate.

Isolation of Glycoprotein from DEAE-Cellulose Fraction I. Polyacrylamide disc electrophoresis (Davis 1964) of fraction I from DEAE-cellulose column revealed the presence of several amido black positive bands, only one of which, the slowest moving, was also periodic acid–Schiff positive (Figure 2). The periodic acid–Schiff positive component was resolved from the rest of the protein contaminants by filtration of fraction I through a Sephadex G-100 column. The glycoprotein eluted as a single peak which was slightly retarded. The peak was dialyzed and lyophilized. The yield of this glycoprotein, designated thereafter as porcine valvular glycoprotein I, was approximately 4.1 mg/100 g of wet valvular tissue.

Criteria of Homogeneity and Estimation of Molecular Weight of Porcine Valve Glycoprotein I. The physicochemical homogeneity and molecular weight of the purified glycoprotein was assessed by subjecting it to polyacrylamide disc electrophoresis in several buffer systems, analytical isoelectric focusing, and Sephadex G-150 filtration.

Results of polyacrylamide disc electrophoresis (Figure 2) reveal that electrophoresis in the discontinuous buffer system of Davis (1964), with 0.1% sodium dodecyl sulfate added, continuous buffer system of Peacock et al. (1965), and sodium dodecyl sulfate buffer system of Weber and Osborn (1969) without addition of 2-mercaptoethanol to the sample, gave a single amido black positive band which was also periodic acid–Schiff positive. Polyacrylamide disc electrophoresis of glycoprotein in sodium dodecyl sulfate buffer system of Weber and Osborn (1969), which contains 2-mercaptoethanol, resulted in the appearance of several amido black and periodic acid–Schiff positive bands (Figure 2). A similar banding pattern was obtained when the glycoprotein was first reduced with 2-mercaptoethanol in the presence of 4 M guanidine-HCl and then subjected to polyacrylamide disc electrophoresis in sodium dodecyl sulfate buffer. From these results it was concluded that reduction of this glycoprotein results in appearance of several components previously held together by S–S linkages.

Analytical isoelectric focusing resulted in the appearance of a single band pI = 5.8 (Figure 3). An approximate molecular weight of 120 000 was obtained by subjecting porcine valve glycoprotein I to filtration through a calibrated Sephadex

TABLE II: Amino Acid and Carbohydrate Composition of Porcine Valvular Glycoprotein I and Porcine Valvular Glycoprotein III.

	Porcine Valvular Glycoprotein I		Porcine Valvular Glycoprotein III[h]	
	Residues/Mol[a]	Nearest Integral Residue	Residues/Mol[b]	Nearest Integral Residue
Amino Acids				
Lysine	58.2 ± 2.66	58	38.9 ± 0.88	39
Histidine	19.8 ± 0.64	20	12.7 ± 0.64	13
Arginine	37.9 ± 2.46	38	12.7 ± 0.74	13
Aspartic acid	73.8 ± 1.44	74	53.8 ± 1.66	54
Threonine[c]	71.5	71	27.8	28
Serine[c]	82.4	82	24.4	24
Glutamic acid	87.8 ± 4.76	88	71.5 ± 1.12	71
Proline	68.7 ± 1.91	69	28.6 ± 0.70	29
Glycine	63.4 ± 2.43	63	23.8 ± 0.57	24
Alanine	56.8 ± 1.36	57	46.4 ± 0.63	46
Half-cystine[d]	13.9	14	28.4	28
Valine[e]	77.8	78	28.8	29
Methionine	1.8 ± 0.11	2	2.4 ± 0.05	2
Isoleucine	25.9 ± 0.76	26	23.9 ± 0.84	24
Leucine	61.8 ± 1.35	62	58.8 ± 1.09	59
Tyrosine	29.7 ± 0.79	30	21.6 ± 0.46	22
Phenylalanine	30.3 ± 1.15	30	25.5 ± 0.71	25
Tryptophan[f]	15.6	16	4.9	5
Amide nitrogen	57.8	58	15.3	15
Carbohydrates				
Fucose	4.6 ± 0.37	5	0.4 ± 0.02	1
Mannose	9.8 ± 0.53	10	1.7 ± 0.13	2
Galactose	14.8 ± 0.62	15	2.9 ± 0.23	3
Glucose	11.7 ± 0.40	12	3.1 ± 0.16	3
Glucosamine	6.8	7	3.7	4
Galactosamine	1.8	2	Nil	
Sialic acid[g]	Nil		0.7	1

[a] Based on a molecular weight of 120,000. [b] Based on a molecular weight of 72,000. [c] Extrapolated to zero time hydrolysis. [d] Determined as cysteic acid following performic acid oxidations. [e] 48 h hydrolysis. [f] Determined spectrophotometrically. [g] Values expressed as *N*-acetylneuraminic acid. [h] Analytical isoelectric focusing results in the detection of a minor and major band. For details see text.

G-150 column.

Chemical Composition of Porcine Valve Glycoprotein I. The amino acid and carbohydrate composition of the porcine valve glycoprotein I is given in Table II. Large amounts of acidic and hydroxy amino acids were present. Analysis of amide nitrogen indicated that about one-third of acidic amino acids were in the form of asparagine and glutamine, which helps explain the behavior of this glycoprotein on DEAE-cellulose column and its near-neutral isoelectric point. It contained fucose, mannose, galactose, glucose, glucosamine, and galactosamine in molar ratios of 5:10:15:12:7:2 per mole of glycoprotein. The glycoprotein contained no sialic acid. It was devoid of any hexuronic acid, hydroxyproline, hydroxylysine, and sulfate.

Identification of Glycoproteins in Fraction II from DEAE-Cellulose. Polyacrylamide disc electrophoresis (Davis, 1964) of DEAE-cellulose fraction II resulted in the identification of two major and one minor amido black positive bands, which were also periodic acid–Schiff positive (Figure 2). Attempts to resolve these glycoproteins individually using ion-exchange and molecular-sieve column chromatography were unsuccessful. The glycoproteins eluted in this peak are designated as porcine valvular glycoprotein II complex.

Isolation of Glycoprotein from DEAE-Cellulose Fraction III. Polyacrylamide disc electrophoresis of fraction III in the discontinuous system of Davis (1964) gave a single amido black positive band which was also periodic acid–Schiff positive (Figure 2). The yield of this glycoprotein, designated thereafter as porcine valve glycoprotein III, approximated 4.5 mg/100 g of wet valvular tissue.

Criteria of Homogeneity and Estimation of Molecular Weight of Porcine Valve Glycoprotein III. The purity was further assessed by subjecting it to polyacrylamide disc electrophoresis in two additional buffer systems, those of Peacock et al. (1964) and Weber and Osborn (1969). In all instances it gave a single amido black positive band, which was also periodic acid–Schiff positive. Results of sodium dodecyl sulfate disc electrophoresis (Weber and Osborn, 1969) indicated that the molecular weight of valve glycoprotein III was approximately 72,000.

Filtration of valve glycoprotein III through a calibrated Sephadex G-100 column resulted in the elution of a single symmetrical peak in the region of molecular weight of approximately 72 000.

Analytical isoelectric focusing resulted in the detection of two bands, a major band with an isoelectric point at pH 4.1 and a less prominent component which banded at pH 4.3 (Figure 3). The resolution of the porcine valve glycoprotein III into two bands with analytical isoelectric focusing reflects the high sensitivity of this technique, since both the polyacrylamide disc electrophoresis and Sephadex filtration studies resulted in detection of a single component.

Chemical Composition of Porcine Valve Glycoprotein III. The amino acid and carbohydrate composition of the glycoprotein is summarized in Table II. The glycoprotein was rich in acidic amino acids and poor in amide nitrogen, a finding which is consistent with its behavior on DEAE-cellulose column and its low isoelectric point. It contained one residue each

of fucose and sialic acid, two residues of mannose, three residues each of galactose and glucose, and four residues of glucosamine per mole of glycoprotein. The glycoprotein was devoid of hexuronic acid, hydroxyproline, hydroxylysine, and sulfate.

Nature of Carbohydrate–Peptide Linkage of Porcine Valve Glycoprotein I and Porcine Valve Glycoprotein III. Preliminary studies regarding the nature of the carbohydrate–peptide linkage suggest that the linkage is stable to alkaline hydrolysis under conditions described by Baig and Aminoff (1972). The carbohydrate–peptide linkage may therefore be *N*-glycosidic, in which the carbohydrate moiety is linked to amide N of asparagine or glutamine residues of the peptide backbone.

Comparative Studies of Porcine Valve Glycoprotein III and Porcine Aortic Intimal Glycoprotein. The chemical composition and molecular weight of these two glycoproteins suggested that they were similar. To examine this further, porcine valve glycoprotein III and aortic intimal glycoprotein (courtesy of Dr. Wagh) were subjected to polyacrylamide disc electrophoresis. The two glycoproteins were similar, as indicated by their similar electrophoretic mobilities in parallel gels using several buffer systems and the occurrence of only one band when the two glycoproteins were coelectrophoresed on the same gel. Analytical isoelectric focusing of the aortic intimal glycoprotein also yielded one major and one minor band, similar to that observed for porcine valve glycoprotein III (Figure 3).

Discussion

The study of the physicochemical and biological properties of mesenchymal glycoproteins requires their isolation from tissue in a form most similar to their native form. To achieve this, mild extraction and isolation techniques described by Radhakrishnamurthy et al. (1964) and Wagh and Robert (1972) were utilized; these resulted in the extraction of four and isolation of two of these major mesenchmyal glycoproteins from porcine mitral valves.

The finding that valve glycoprotein III is similar to the aortic intimal glycoprotein isolated by Wagh and Roberts (1972) suggests that this glycoprotein is common to both tissues. Although homogeneity of this glycoprotein was initially suggested by several physical criteria, analytical isoelectric focusing resulted in the detection of a major and a minor component. Our studies also provide additional data to that previously reported by Wagh and Roberts (1972) regarding the nature of this glycoprotein. These include the absence of subunit structure, the identity of amino sugar as glucosamine, and the low amide nitrogen content.

The unique chemical feature that was encountered for both porcine valve glycoprotein I and porcine valve glycoprotein III was the presence of large amounts of glucose in their carbohydrate moieties. It is of interest that the glycoproteins previously isolated from other mesenchymal tissues (Radhakrishnamurthy et al., 1964; Barnes and Partridge, 1968; Moczar and Robert, 1970; Wagh and Roberts, 1972) also contain glucose. The well-known biological substances in which glucose is present are glycolipids and collagen. Glycolipids move rapidly toward the anode upon polyacrylamide disc electrophoresis and are usually found close to the region of the bromophenol blue dye marker (Evans and Curd, 1971). Neither valvular glycoprotein I nor valvular glycoprotein III showed this characteristic. Furthermore, the extraction of aqueous solutions of the two glycoproteins with chloroform:methanol resulted in complete recovery of the carbohydrate components in the aqueous phase. Evidence for the lack of contamination of the glycoprotein preparations by collageneous material is supported by the absence of hydroxyproline and hydroxylysine and the precipitation of these two glycoproteins with 5% trichloroacetic acid with complete recovery of the carbohydrates in the precipitates.

Recent reports have suggested that antigenic cross-reactivity of streptococcal and tissue components could play a role in the pathogenesis of rheumatic heart disease. Studies by several investigators (Goldstein et al., 1968; Ayoub, 1972; Kawakita and Iwamoto, 1975) have implicated the antigenic cross-reactivity between the streptococcal group A carbohydrate and of structural glycoproteins of mitral tissue in the pathogenesis of valvular heart disease. In addition, porcine mitral valves have become the most commonly used heterografts for replacement of damaged valves in humans. For these reasons, our extraction of valvular glycoproteins using mild techniques, characterization of the physicochemical properties of two major glycoproteins, and establishment of the common presence of one of these glycoproteins in both mitral tissue and aortic intima assume added significance.

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A Nuclear Magnetic Resonance Study of the Heme Environment in Beef Liver Catalase†

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ABSTRACT: The effect of high-spin heme iron in beef liver catalase on the longitudinal and transverse proton relaxation rates of the solvent has been used to probe the environment of the paramagnetic center. The longitudinal proton relaxation rates were measured as a function of temperature (5–31 °C), frequency (5–100 MHz), and pH. T_{1p} was found to be pH independent in the range 6–11, indicating that no significant difference occurs in the heme surrounding within this pH range. The ligands formate and acetate, which preserve the spin state of the heme iron upon ligation, displace a water molecule from the sixth coordination position. This reaction is pH independent, while the binding measured by optical spectroscopy is pH dependent. The electron donors methanol and ethanol essentially do not change the proton relaxation rates. The temperature and frequency dependencies indicate that the relaxation times are governed by the electronic relaxation time of the high-spin ferric iron, τ_s. τ_s, which was found to be frequency independent, could not be determined from the T_{1p}/T_{2p} ratio, but only from the frequency dependence of the longitudinal relaxation rate at low frequencies. The results of the least-squares fit of the data to the theory indicate that there is one iron-bound rapidly exchanging water molecule. For the Fe^{3+} ion it was determined that $\tau_s = 7 \times 10^{-11}$ s.

In spite of the extensive data on various physical properties of catalase and its derivatives (Nicholls and Schonbaum, 1963; Deisseroth and Dounce, 1970), the nature of the ligand at the sixth coordination position is not definitely known. Three different proposals have been made for the identity of this ligand. The prevalent assumption is that a water molecule is bound to the catalase heme iron (Nicholls, 1962). This hypothesis was critized on the basis of two experimental facts. First, in contrast to metmyoglobin and methemoglobin, which possess a water molecule at the sixth coordination position (Brill and Williams, 1961; Fabry et al., 1971; Perutz, 1970), catalase does not form alkaline hematin derivatives up to nearly pH 12 (Chance, 1952a), nor are the catalatic and peroxidatic reactions of catalase sensitive to pH changes (Chance, 1952a; Jones and Suggett, 1969). Second, an apparent uptake of a proton results from the reaction between the enzyme and the anions F^- and CN^- (Chance, 1952a,b). These experimental facts were explained by postulating a hydroxide group (Theorell and Paul, 1944; Chance, 1952b) or an amino acid residue of the protein with a high pK (George and Lyster, 1958) as the relevant ligands of the iron in catalase. However, none of the three suggested possibilities was substantiated by direct experimental evidence. In view of these uncertainties an investigation was conducted in order to identify the sixth iron ligand in beef liver catalase by the pulsed NMR technique.[1] It should be noted that there seems to be no other way, including x-ray evidence, of choosing between water and an hydroxyl anion as the species occupying the sixth coordination position.

NMR spectroscopy has the potential of counting the hydration number of a paramagnetic metal ion bound to a biological macromolecule (Navon, 1970; Mildvan and Cohn, 1970; Dwek, 1972). If the various parameters which are responsible for the proton relaxation mechanisms can be precisely evaluated, it is possible to decide whether OH^- or H_2O groups are coordinated to the metal ion (Lanir et al., 1975). The proton relaxation technique has been used several times in attempts to study the accessibility of solvent molecules to hemes in various heme proteins (Maricic et al., 1966; Fabry et al., 1971; Pifat et al., 1973; Lanir and Aviram, 1975; Lanir and Schejter, 1975a) and to probe the immediate environment of the Fe^{3+} ion (Fabry and Eisenstadt, 1974; Lanir and Aviram, 1975; Gupta and Mildvan, 1975). A preliminary account of part of this work has been published (Lanir and Schejter, 1975b).

Materials and Methods

Beef liver catalase was obtained from Boehringer Mannheim GmbH. Three milliliters of the enzyme suspension (1 g/50 ml)

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[1] Abbreviations used are: NMR, nuclear magnetic resonance; zfs, zero field splitting; EPR, electron paramagnetic resonance.