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THE SUBUNIT STRUCTURE OF HEART MUSCLE CHALONES

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SUMMARY: Recently, chalones have been isolated from adult bovine hearts and purified to homogeneity (2). A combination of equilibrium centrifugation in 6.0 M Guanidinium chloride and SDS polyacrylamide gelelectrophoresis of the chalones both under reduced conditions suggest the involvement of four indentical subunits with a molecular weight of about 150 000 each. Electron microscopy of negatively stained preparations showed a globular structure. From the amino acid analysis it is evident that the chalones has a net negative charge and a predominant hydrophobic amino acid composition. Isoelectric focusing shows a pI of 5.1. The sugars detected were, in molar proportions, D-Mannose (3.0), D-Galactose (2.5), D-Glucose (1.0) and D-Glucosamine (1.7).

INTRODUCTION

A number of researchers (reviewed in ref. 1), working with a variety of partially purified chalones or extracts containing chalone-like activities, have suggested a mitotic role for these proteins. However, the precise mechanism of chalones as a specific endogenous mitotic inhibitor is still unclear.

Recently, chalones have been isolated and purified from adult bovine hearts(2). Analytical ultracentrifugation of the highly purified fraction indicated a sedimentation coefficient of 16.7s and a molecular weight of 715 500 daltons. In vivo studies have confirmed that these chalones isolated from adult bovine hearts inhibit DNA synthesis of new-born hamster hearts, when injected intraperitoneally. In addition, it was found to be tissue and not species specific and had no cytotoxic effects on the target cells. In this communication we report the subunit structure and physico-chemical properties of this molecule.

MATERIALS AND METHODS

Isolation and Purification of heart muscle chalones: Chalones were isolated and purified to homogeneity, using ultrafiltration followed by Concanavalin A-Sepharose affinity chromatography and subsequent sucrose density gradient centrifugation essentially as described by Kriek et al (2).

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Analytical Ultracentrifugation: Measurements were made with a Beckman Model E ultracentrifuge equipped with an automatic photoelectric scanner with a 10 inch recorder and electronic speed control. A six place An-G rotor with Kel F centrepieces was used throughout. Unless otherwise stated, lyophilized samples were dialyzed against 0.02M phosphate buffer, pH 7.4, prior to ultracentrifugation.

Molecular weights under non-denaturing conditions were determined by the low speed sedimentation equilibrium technique (3) at 4400 rpm at 17°C and the partial specific volume determined as previously described (4). To determine the half molecular weight of the chalones, a lyophilized sample was dissolved in phosphate buffer and extensively dialyzed against 6.0M Guanidinium chloride (Gdm-Cl). Ultracentrifugation was performed at 20°C with a constant equilibrium speed of 13 000 rpm. For the determination of the smallest molecular weight fraction, chalones were reduced in 6.0M Gdm-Cl for 1 h under nitrogen with a 100-fold excess of dithiothreitol per protein disulfide bond in 0.02M phosphate buffer, pH 7.4 (5-7). The -SH groups were alkylated with a molar excess of 2.5:1 of iodoacetic acid to dithiothreitol by incubating for 15 min. The solution was then dialyzed extensively against 6.0M Gdm-Cl at 4°C. Ultracentrifugation was performed at 20°C with a sedimentation equilibrium speed of 20 000 rpm. Equilibrium was assumed in every case when no detectable difference in two sedimentation patterns taken 8 h apart, could be observed.

Analytical SDS-Polyacrylamide Gel Electrophoresis: Disc gel electrophoresis was carried out essentially according to Shapiro et al (8). The final acrylamide concentration was 4% with 4% of its weight as N,N'-methylenebisacrylamide. The gels contained 0.05M Tris/glycine (pH 8.3) plus 1% SDS. The same buffer was used for electrophoresis at a constant current of 3 mA/tube until the bromophenol blue had reached the bottom of the tube. Reduction of the sample in electrophoresis buffer containing 1% SDS and 1% 2-mercaptoethanol was performed by boiling the solution for 2 min.

Protein concentration determination: Protein concentration was determined by the method of Lowry et al (9), using bovine serum albumin as standard.

Electron microscopy: The samples were negatively stained using phosphotungstic acid (PTA), pH 7.0 and were then picked up on thin carbon support films on 400 mesh copper grids. These were then viewed on a Siemens Elmiskop 102 transmission electron microscope at 80 kV.

Amino acid analysis: Amino acid analyses were performed after hydrolysis in 6.0 N HCl (constant boiling) at 110°C for 24 h in an oxygen-free, nitrogen atmosphere, on a Beckman Model 119 Bl automatic amino acid analyser equipped with a computer output (10).

Determination of the isoelectric point: A lyophilized sample was dissolved in 0.01 M Tris/HCl, pH 7.0, and digested by neuraminidase (Boehringer-Mannheim) at 37°C for 1 h (11), after which it was dialyzed against distilled water and lyophilized. Isoelectric focusing of the neuraminidase treated sample was done essentially as described by Radola (12). The sample was dissolved in distilled water and mixed directly with 5% (W/W) slurry of Sephadex G75 (IEF Grade) and 2% Ampholines (equal quantities of pH 4-6 and pH 5-8 Ampholine). The focusing took place for 16 h at 8 Watts constant power at 20°C. The pH gradient on the gel was monitored by a Radiometer combined surface pH-electrode and thirty individual fractions were recovered from the gel by inserting a grid and scraping out the gel with a spatula. The gel was washed in mini-columns with 0.02 M Tris/HCl, pH 7.4 and the absorbance of the eluates monitored at 280 nm.

Carbohydrate analysis: Sugars were quantitatively analyzed by gas-liquid chromatography as alditol acetates following hydrolysis of the chalones with 2.0 M trifluoroacetic acid (15). Chromatograms were recorded isothermally at 220°C using a Carlo Erba Model 4200 Gas Chromatograph, equipped with a FID Detector and a column (2m x 4mm i.d.) of 3% OV-225 on Chromosorb W (80-100 mesh).

TABLE 1 Molecular weights of chalones from sedimentation equilibrium centrifugation. Time to reach equilibrium was 50 h.

Protein concentration (mg/ml)	Equilibrium speed (rpm)	Molecular weight
1.0	4400	675 000
0.7	4400	723 000
0.6	4400	722 000
0.5	4400	720 000
0.3	4400	737 500
	Average	715 500

A constant flow rate of 40 ml per min of nitrogen was maintained, with an inlet temperature of 250°C and a detector temperature of 300°C. Mass spectra were obtained at 70 eV on a VG Micromass 16 F spectrometer fitted with a VG 2000 data system and linked to the Gas Chromatograph.

RESULTS AND DISCUSSION

The molecular weights obtained by low speed equilibrium centrifugation at various protein concentrations are summarized in Table 1. An average molecular weight of 715 500 dalton was calculated using the date from the linear ln c versus r^2 plots and a partial specific volume of 0.720 ml/g as determinded by substituting the slopes obtained in different H_2O/D_2O ratios in the appropriate relationship of Edelstein and Schachman (4). When equilibrium centrifugation of the chalone was carried out in the presence of 6 M Gdm-Cl, the average molecular weight was reduced to 395 000 (Table 2). When equilibrium centrifugation was performed on reduced and alkylated chalones, an average molecular weight of 150 000 was obtained (Table 3). The linear relationship between ln c versus r^2 (Fig 1), demonstrates the molecular homogeneity and thus the smallest molecular weight subunit.

The polypeptide composition of the unreduced and reduced 16.7s chalone as analyzed on SDS-polyacrylamide gel electrophoresis is presented in Fig. 2. The electrophoretic pattern of the unreduced and reduced protein showed only one band in each case an may represent the chalone dimer and monomer, respectively.

TABLE 2 Molecular weight analyses of chalones by sedimentation equilibrium centrifugation in 6.0 M Gdm-Cl. Time to reach equilibrium was 50 h.

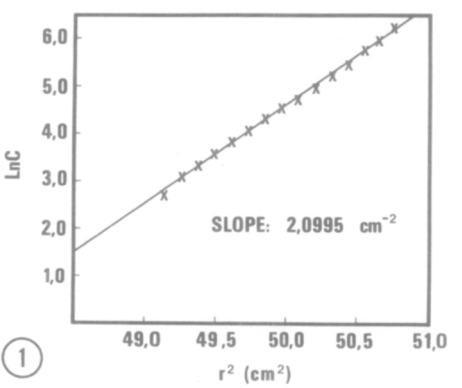
Protein concentration (mg/ml)	Equilibrium speed (rpm)	Molecular weight
0.7	13000	392 000
0.6	13000	375 500
0.5	13000	401 500
0.3	13000	403 500
0.2	13000	402 500
	Average	395 000

TABLE 3 Sedimentation equilibrium data of reduced and alkylated chalones. Time to reach equilibrium was 52 h

Protein concentration (mg/ml)	Equilibrium Speed (rpm)	Molecular weight
0.5	20 000	148 000
0.4	20 000	156 000
0.2	20 000	146 000
	Average	150 000

The homogeneity in band composition of the reduced protein indicated the involvement of identical subunits in the composition of the chalone. These results together with the analytical ultracentrifugation data suggest that the chalone molecule consists of four identical subunits with an average molecular weight of about 150 000 each.

A model for the chalone molecular structure is proposed in which the half molecules are linked by noncovalent interactions. Each half molecule in turn is composed of two equal-sized units coupled by disulphide bonds. Some half molecules, however, may be linked by noncovalent bonds as is evident from SDS-polyacrylamide gelelectrophoresis. The unreduced sample shows a second band near the top of the gel (Fig 2) with a size corresponding to the undissociated molecule. The amount of this second band never exceeded 10% of the total coomassie



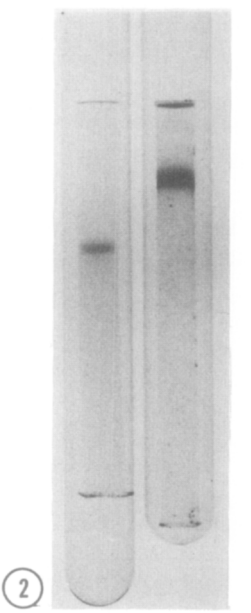


Fig. 1 The ln c versus r^2 plot obtained from equilibrium centrifugation of reduced alkylated chalones in 6M Gdm-Cl.

Fig. 2 Electrophoretic analysis of reduced (left) and unreduced (right) 16.7s heart muscle chalone.

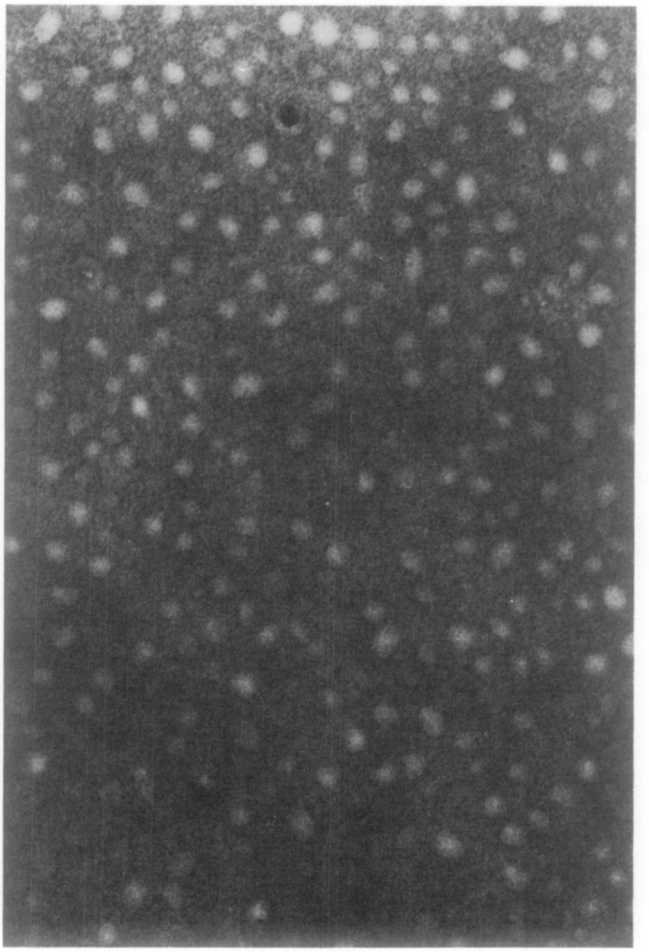


Fig. 3 Negatively stained chalones with 2% PTA. Magnification x 100 000.

blue stainable protein. The presence of a small amount of undissociated molecules in 6M Gdm-Cl may explain the somewhat high weight average molecular weight of about 400 000 for the half molecule as determined by sedimentation equilibrium.

Negatively stained preparations of highly purified chalones showed a globular structure (Fig 3).

The amino acid analysis is shown in Table 4. It is evident from the amino acid composition that the heart muscle chalone has a net negative charge and a predominant hydrophobic amino acid composition.

Prior to isoelectric focusing, the chalone was first digested by neuraminidase to remove sialic acid residues, since the charged groups confer microheterogeneity to macromolecules (11, 13). An approximate increase in pI of 0.2 to

TABLE 4 Amino acid composition of the 16.7s heart muscle chalone[a]

Amino acids	Residues/1000
Aspartic acid	75.7
Threonine	69.1
Serine	85.6
Glutamic acid	118.9
Proline	44.2
Glycine	58.5
Alanine	76.0
Citruline	8.2
Valine	100.7
Cysteine	32.3
Methionine	20.3
Isoleucine	28.2
Leucine	81.8
Tyrosine	38.7
Phenylalanine	47.4
Tryptophan	0.6
Ornithine	0.07
Lysine	54.1
Histidine	22.2
Arginine	36.5

[a]Average of duplicate analyses performed on hydrolysates of 1 mg protein each.

0.6 pH units may be expected if the sialic acid were present (13). An elution profile (Fig 4) of chalone after isoelectric focusing, shows a single band of protein at a pI of 5.1. This is to be expected, since the amino acid composition indicated an excess of acidic over basic residues. The approximate number of amidated carboxyl groups per subunit was calculated using the obtained pI and amino acid composition data and intrinsic dissociation constants of titratable

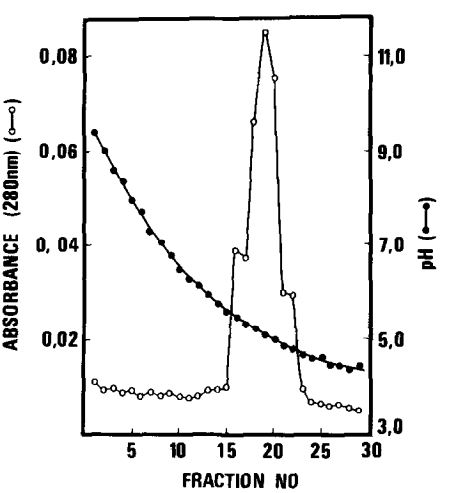


Fig.4 Isoelectric focusing of neuraminidase treated chalone.

TABLE 5 Sugar analysis of heart muscle chalone[a]

Sugar	Mole Percent
D- Mannose	37
D-Galactose	30
D-Glucose	12
D-Glucosamine	21

[a] Average of duplicate analyses.

groups in randomly coiled proteins (14). The number is the excess of aspartate and glutamate residues after neutralization of the positive charges contributed by lysine, arginine and histidine. This is approximately 24% of the total acidic residues.

The neutral and amino sugars released upon acid hydrolysis of the chalones were identified by paper chromatography and by a combination of gas-liquid chromatography and mass spectrometry of the derived alditol acetates. The sugars detected in this way (Table 5) were D-Mannose (3.0), D-Galactose (2.5), D-Glucose (1.0) and D-Glucosamine (1.7) in the molar proportions shown. Preliminary studies (by methylation analysis) have indicated that these sugars (comprising approximately 24% of the total molecular weight of the chalone) are present as terminal groups or as short carbohydrate chains.

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