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HYDRODYNAMIC AND CIRCULAR DICHROIC ANALYSIS OF MAMMARY-DERIVED GROWTH INHIBITOR (MDGI)

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SUMMARY: The mammary-derived growth inhibitor exists in solution as a monomeric molecule with a molar mass of 14 500 ± 400 g/mol. The largest diameter and the height of the polypeptide chain were estimated to be 3.75 ± 0.25 nm and 2.01 ± 0.13 nm, respectively. This is in good agreement with the structurally related bovine peripheral myelin P2 protein (about 70 % amino acid sequence homology). CD measurements have revealed MDGI to be a protein with about 50 % ß structure and less than 20 % α helix similarly as in fatty acid-binding proteins. Removal of endogenous long-chain fatty acid by lipidex or storage in the frozen state lead to a destabilization of the active MDGI conformation which is accompanied by a loss of its activity with regard to growth inhibition of Ehrlich Ascites cells.

Recently a purified polypeptide from lacting bovine mammary gland was found to inhibit the growth of mammary epithelial cells in vitro (1,2). This protein was mamed "mammary-derived growth inhibitor" (MDGI) (3). Polyclonal rabbit antiserum against MDGI cross-reacts with bovine heart fatty acid-binding protein (FABP) and bovine peripheral myelin P2 protein (4), which is in accordance with the sequence homology of the protein (3). MDGI also binds fatty acids (5). So far, the functional meaning of fatty acid binding has not been elucidated for the homologous proteins. A carrier function has been suggested (6). Alternatively, hydrophobic ligand could stabilize a functionally important conformation. In a first attempt this has been investigated in the present paper. Hydrodynamic and CD measurements were performed to elucidate structural properties of MDGI of higher structure levels. The results suggest a close structural relationship of MDGI with the proteins myelin P2 (7) or intestinal FABP (8) at these structure levels, too. We interpreted the data in relation to the fatty acid-binding and the cell growth inhibitory effect of MDGI.

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MATERIALS AND METHODS

MDGI was prepared and purified as reported in (3). The samples in PBS buffer (20 mM sodium phosphate, 150 mM NaCl, pH 7.4) were either directly used or frozen in liquid nitrogen and stored at -20 °C until use. The protein content was determined from the absorption coefficient $A_{280\ nm}$ = 10.63 obtained for a 1 % protein solution.

Delipidization of MDGI was performed by treatment with Lipidex 1000 (Packard) at 37 °C as described by Dahlberg et al.(9). In brief, MDGI (ca. 40-120 µg in 500 µl) was incubated with 500 µl (sedimentated gel bed) Lipidex 1000, previously equilibrated with PBS, at 37 °C for 30 min. The mixture was then centrifuged and the supernatant carefully aspirated. Under these conditions, at least 90 % of [^{3}H]-labelled palmitic acid, precomplexed to MDGI (5), were removed from the protein.

Hydrodynamic studies were carried out with an analytical ultracentrifuge Spinco E (Beckman) equipped with ultraviolet absorption optics, monochromator and scanner. For diffusion experiments a capillary type double-sector synthetic boundary cell at 6000 rpm was used. The diffusion coefficients (D) were measured with regard to the time dependent broadening of the boundary registered at 280 nm and corrected to the viscosity of water and 20 °C. Furthermore, within the same experiment sedimentation coefficients (s) of the samples were determined at rotor speeds of 40,000 rpm. The values obtained were corrected for density and viscosity of water at 20 °C.

The partial specific volume ($\bar{v}$) was calculated from the amino acids composition (3), and the apparent specific volume increments of amino acids according to Cohn and Edsall (10). The molecular mass (M) of MDGI was calculated either from s, D and $\bar{v}$ using the Svedberg formula or by the sedimentation equilibrium technique. Information about the volume (V), Stoke s radius (R) and the shape or frictional ratio (f/f_o) of MDGI were obtained by the formulas (1-3), respectively.

$$V = M \cdot \bar{v}/N_A \qquad (1)$$

$$R = \frac{M(1-\rho\bar{v})}{(f/f_o)\,6\pi s\eta N_A} \qquad (2)$$

$$f/f_o = 10^{-8}\left(\frac{1-\rho\bar{v}}{D^2 \cdot s \cdot \bar{v}}\right)^{1/3} \qquad (3)$$

where N_A is the Avogadro number, ρ and η are solvent density or viscosity, respectively. The frictional ratio, besides the shape dependent moiety still contains a portion contributed by the hydration $(f/f_o)_{hydr}$. This value can be calculated from the amount of water (W) bound at the protein according to equation (4)

$$(f/f_o)_{hydr.} = \left(1 + \frac{W}{\rho\bar{v}}\right)^{1/3} \qquad (4)$$

Assuming MDGI as an oblate ellipsoid of revolution with (a) the large and (b) the small half axis, the latter one can be calculated from equation (5)

$$b = Ro \cdot \tan^{-1} \left[(a^2/b^2)-1\right]^{1/2} / \left[(a^2/b^2)-1\right]^{1/2} \quad (5)$$

with

$$Ro = R/(f/f_o)_{hydr.} \quad (6)$$

The circular dichroism measurements were carried out on a Roussel-Jouan Dichrograph CD 185 at protein concentrations of about 0.3 g/l in cells with 1 mm path length. For dilution experiments the thickness of cells was increased. The estimation of secondary structure has been done on a computer using the program CONTIN VERSION 2DP according to Provencher and Glöckner (11).

RESULTS

Hydrodynamics

MDGI is characterized by low sedimentation coefficients of about 2 S. The values obtained from different samples or preparations sometimes vary considerably (Fig. 1a). In deluted solutions containing 20-50 µg protein/ml the sedimentation coefficients of freshly prepared (active) MDGI amount to 1.76 ± 0.02 S. Diffusion

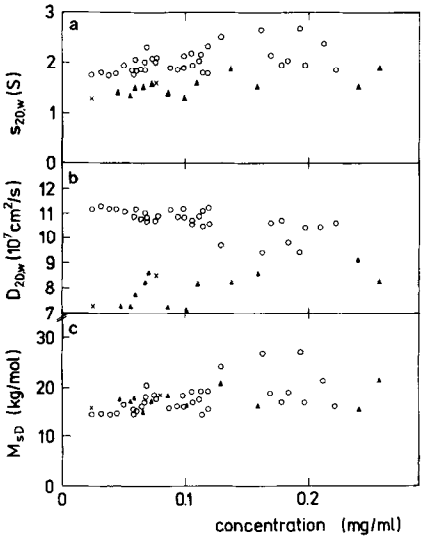

Fig. 1: Concentration dependence of sedimentation coefficients (a) diffusion coefficients (b) and molar mass (c) of freshly prepared (o) MDGI in PBS. (x) Corresponding values after treatment of MDGI with lipidex or (▲) MDGI samples, stored for more than 3 months in the frozen state

Table 1: Physical properties of MDGI

Parameter	value
Sedimentation coefficient ($S_{20,w}$)	1.76 ± 0.02 S
Diffusion coefficient ($D_{20,w}$)	$(11.3 \pm 0.2)10^{-7}$ cm^2/sec.
Molar mass (M_{sD})	14 650 ± 400 g/mol
Molar mass (M_{equi})	14 350 ± 300 g/ml
Frictional ratio (f/f_o)	1.15_8
Partial specific volume ($\bar{v}$)	0.742_2 cm^3/g
Stoke's radius (R)	1.89_5 nm
Dry volume (V)	18.04 nm^3
Largest diameter (d)	3.75 ± 0.25 nm
Height (h)	2.01 ± 0.13 nm

measurements of the same samples result in values of $(11.3 \pm 0.2)10^{-7}$ cm^2/sec, which decrease slowly in higher concentrations (Fig. 1b). From both parameters in combination with the partial specific volume of 0.742_2 cm^3/g the molecular mass of MDGI was calculated (fig. 1c). The data of MDGI in solution vary between 14 000 and 20 000 with a mean value of 14 650 ± 400 in strong dilution. Using the sedimentation equilibrium technique the molecular mass was determined to be 14 350 ± 300. Both values are in good agreement with that obtained from the amino acids sequence (14 450). Furthermore, some MDGI solutions should contain oligomeric molecules besides of the monomeric form. These observations are valid independently of whether freshly prepared samples or samples stored briefly up to two weeks were used. From the hydrodynamic mobility given by the data of s and D further molecular parameters of MDGI were calculated (see Tab. 1). They were obtained by means of equation (1-6) considering an average amount of 0.3 g bound water/g protein. Taking into account an oblate ellipsoid of revolution, dimensions of 1.07 nm for the small and 2.00 nm for the large half axis were determined. Similar data, 1.75 nm for the radius and 1.875 nm for the height, were calculated considering MDGI as a flat cylinder. So, the average dimensions of MDGI should be 3.75 ± 0.25 nm for the largest diameter and 2.01 ± 0.13 nm for the height of the molecule. In order to remove endogenous fatty acids MDGI was treated with Lipidex 1000, which to some extent resulted in aggregation of the protein, indicating a decreased stability of the dissolved molecules. Hydrodynamic analysis of delipidated soluble MDGI revealed

a reduced hydrodynamic mobility (see Fig. 1 a,b). On the other hand, the molecular mass is comparable with the "native" fatty acid-containing MDGI: The frictional ratio (f/f_0) of delipidated samples is increased to about 1.40 and more indicating a larger surface or partial unfolding of the polypeptide. A comparable behaviour in the hydrodynamic data was observed for MDGI samples which were stored 3 months and longer in the frozen state (Fig. 1).

Secondary structure

The CD spectrum of MDGI shows a large negative ellipticity between 216 and 218 nm indicating a high amount of ß structure (results not shown). The analysis of the CD spectrum for prediction of secondary structure according to (11) reveals 17 % α helix, 49 % ß strands and 34 % reversed turns or random coil, respectively. Samples which were stored a few months in the frozen state show similar values. However, the content of ß strands is somewhat reduced in favour of the unordered or remainder structure.

Activity of MDGI

The inhibitory effect on the growth of Ehrlich Ascites cells was measured at nanomolar concentrations of MDGI, depending on the protein concentration and reaching a plateau at 10 ng/ml. Samples which were stored for several months in the frozen state lost their activity. Apart from some aggregates, the inactive MDGI is a monomeric molecule but with partial unfolding (increasing frictional ratio f/f_0 to about 1.40 and larger).

DISCUSSION

Comparison of the structural data obtained in this study for MDGI with those of the homologous proteins reveals a striking similarity. Besides the similar molar mass, MDGI seems to have a similar shape as myelin P2 with a diameter of 3.5 nm and a height of about 2 nm (7). This is not surprising when considering a sequence homology of about 70 % between both proteins. The structural relationship to the intestinal FABP of rat (8) with only 32 % of identical amino acids indicates that structural similarities are determined by distinct amino acids rather than by overall homologies. This is valid for a family of proteins sharing the ability to bind fatty acids, retinoic acid or prostaglandin (12-16). Analyzing CD spectra of different FABP with the program of

Table 2: Secondary structure of MDGI and some related proteins calculated from CD-data according to (11)

Substance	α-helix (%)	ß-strand (%)	rev.turn (%)	random coil (%)	Reference
MDGI	17	49		34	this paper
FABP (bovine heart)	15	51		34	(6)
FABP (pig heart)	19	48	8	25	(17)
FABP (human heart)	25	45		30	(18)
FABP (bovine liver)	12	46	15	27	(19)

Provencher and Glöckner (11) a good relation between these proteins (6, 17-19) and MDGI is observed (Tab. 2) in spite of the limited exactness of such calculating procedures. Comparing these data with those of myelin P2 (7), obviously the amount of ß structure is higher at the expense of the random coil

The low stability of MDGI preparations with regard to inhibitory activity under different storage conditions (2) has hampered the functional investigation of this protein. MDGI preparations inactivated by storage exhibited unchanged molecular mass when analyzed by gel electrophoresis in the presence of SDS, unchanged lipid binding properties and little or unchanged antigenetic activity (F.-D. Böhmer et al. unpublished observations). Interestingly, the hydrodynamic studies outlined in this paper suggested structural changes of the MDGI molecules upon storage, which are similar to the change obtained after delipidization, i.e. they suggest a partial unfolding with enlargement of the surface. From the strong structural similarity of MDGI to myelin P2 it is highly suggestive to assume that, similarly as in myelin P2 (7) or FABP (8), long chain fatty acids or related ligands are bound in the interior of a ß barrel in MDGI by hydrophobic amino acids. Presumably, these ligands impart to the MDGI a distinct stability necessary for its interaction with the still unknown receptor in the inhibitory process

Because the difference in the CD spectra between fatty acid bound and free MDGI or bovine liver FABP (19) is either very small or not detectable, the change in the tertiary structure should be directed more into the turn region between the two

ß sheets (see turn between strand D and E in myelin P2 (7)). A proposal for the stabilizing role of fatty acids for this part of the molecule has given by us recently (R. Süßmilch et al. submitted). A stabilizing rather than metabolizing function of bound fatty acids could explain the low degree of specificity with regard to fatty acid binding by FABP s in tissues of clearly different requirement for fatty acids, such as heart or mammary gland. Similar mechanisms, i.e. a partial unfolding of the molecule due to a loss or decay of endogenous lipid ligands might contribute to the inactivation of inhibitory activity of MDGI preparations. A regulation principle based on concentration dependent association as observed in pig heart FABP (17) does not seem to be valid for MDGI. A small increase in the molecular mass of MDGI might be due to the formation of some aggregates. No calculable molar mass concentration relation was found as observed also for liver FABP (20).

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