ON THE SUBUNIT STRUCTURE OF CROTOXIN: HYDRODYNAMIC AND SHAPE PROPERTIES OF CROTOXIN, PHOSPHOLIPASE A AND CROTAPOTIN

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Received July 14,1975

Summary: This paper reports physical-chemical properties of the subunit structure of crotoxin, phospholipase A and crotapotin. The native crotoxin has a sedimentation coefficient of 3S and a radius of gyration of R_g = 16.5 Å and a molecular weight of 30,900. Dissociation of the 3S particle results in two proteins of unequal size with sedimentation coefficients of 1.5 S (crotapotin) and 1S (phospholipase A). These dissociated species and the reconstituted complex were investigated by means of hydrodynamic methods including small angle X-ray scattering. The actual frictional ratios were obtained indicating that crotoxin is a sphere with a Stokes' radius of R_o = 22.5 Å and an axial ratio of 1:3, whereas phospholipase A, depending on the degree of association, has a radius of gyration of R_g = 32.4 Å and a high axial ratio of 1:14 for the monomer. Crotapotin has a radius of gyration of R_g = 12.4 Å, indicating an oblate ellipsoid of revolution of an axial ratio of 1:4. Evidently, the crotoxin complex consists of one highly asymmetric molecule (phospholipase A) and an oblate ellipsoid (crotapotin), which reconstitutes to a spherical 3S-particle (crotoxin).

Phospholipase A (E.C. 3.1.1.4) and crotapotin are the main toxic compounds of the crotoxin complex of Crotolus terrificus [1]. They have been separated and purified by carboxymethyl cellulose chromatography [2, 3]. The acidic protein, crotapotin, with a pK_i = 4.8, has neuro-toxic activity, whereas phospholipase A, pK_i = 9.7, has hemolytic activity. It was found [4] that crotapotin lacks the toxicity and the enzymatic activity of crotoxin but is capable of potentiating the toxicity and inhibits the enzymatic activity of the phospholipase A. Almost nothing is known about the tertiary

and quarternary structure as well as the shape of the components of the crotoxin complex and the crotoxin complex itself. We report here the first experimental data of the shape properties of crotapotin, phospholipase A and the reconstituted complex crotoxin under physiological conditions.

MATERIAL AND METHODS

Crotoxin, phospholipase A and crotapotin was prepared according to [2, 5] . The lyophilized material was dissolved in double distilled water and dialyzed against the appropriate buffers at 4°C for 12 hours. The buffers used throughout this investigation are:

A : 0.01 M CH_3COONa, pH 5.5, 0.06 M KCl,
0.01 M $MgCl_2$, 6 mM ß-mercaptoethanol,

B : 0.01 M TRIS-HCl, pH 8.5, 0.01 M KCl,
0.005 M $MgCl_2$.

Protein concentrations ranged from 5-10 mg/ml for sedimentation velocity runs with Schlieren optics, for viscosity measurements, and for small angle X-ray scattering experiments in solution. Polypeptide chain molecular weights were determined under native conditions and in the presence of 4 M guanidinium-hydrochloride by the sedimentation equilibrium method according to Yphantis [6] in a Model E Beckman Spinco analytical ultracentrifuge equipped with a photoelectric scanner and a RTC-temperature control. The Stokes' radius was determined by gel chromatography (Sephadex G 75) from a logarithmic plot of R_s versus elution volume ($erf^{-1}(1-K_{ol})$) [7] after calibration of the column with standard proteins of spherical shape. Small angle X-ray scattering experiments in solution were performed in a Kratky-camera, equipped with an electonically programmed step scanning device (Müller-Seifert) using monochromatized (bent quartz monochromator) CuK_α radiation. Scattering curves were recorded for different concentrations and temperatures as well as for the buffers. The apparent radius of gyration (R_g) was determined by plotting the logarithm of the scattered intensities against $(2\vartheta)^2$ in the 0.9 I_o to 0.6 I_o region where one normally obtains a straight line. The slope of this

line is proportional to the apparent R_g^2. The values of the apparent R_g were plotted against concentration and extrapolated to infinite dilution. Viscosities were measured in a special designed Ostwald viscosimeter at 4°C with flow times of 145 sec for water.

RESULTS

From sedimentation, diffusion and viscosity experiments the shape of the components of crotoxin, phospholipase A and crotapotin, as well as the reconstituted complex is estimated from the ratio of the frictional coefficients, f/f_o, Simha-parameter ν [8], and the radius of gyration. According to table 1 only crotoxin and crotapotin can be described as spherical in shape, whereas phospholipase A, due to its high axial ratio, is highly asymmetric. Phospholipase A from crotoxin undergoes a reversible equilibrium between a dimer and a tetramer [9] which is governed by i) the concentration of phospholipase A, ii) the pH, and iii) the ionic strength. The stable form is the tetramer from which we obtain the hydrodynamic data listed in table 1; for the monomer of phospholipase A the data are listed in tables 1, 2 and 3. Even from these data it can be seen that the shape of the tetramer in solution is not spherical. The ratio of the long to short axis of an ellipsoid of revolution equivalent to the one of a tetramer that would have the molar frictional ratio of f/f_o is obtained by [10] by means of the Perrin function [11]. In conjunction with Oncley's functions we can get an estimate of the degree of hydration, e.g., the real asymmetry of the polypeptide chain in solution. Values of the degree of hydratation were obtained from small angle X-ray scattering and were found to be 0.30 g H_2O per 1 g phospholipase A. Under the assumption that $\bar{V}$, the partial specific volume, represents the volume occupied by 1 g protein, and the hydrodynamic equivalent has the appropriate volume we obtain cell dimensions for the tetramer of phospholipase A of L = 140-150 Å and with a diameter of D = 11.0 Å. The Simha-parameter of 21.2 for the monomer of phospholipase A is considerably larger than 2.5 for suspended spheres and thus indicates an extended shape. Under the assumption that the

Table 1.

Hydrodynamic properties of crotoxin, phospholipase A and crotapotin, obtained by diffusion, sedimentation velocity and equilibrium experiments.

	crotoxin	phospholipase A		crotapotin	
M_w	30,900[1]	15,400[2]		12,600[1]	
$S_{20,w}$	3.09	1.06[1] 4.30	monomer tetramer	1.46	sedimentation coefficient
$D_{20,w}$	8.98×10^{7}	2.34 6.49	monomer tetramer	13.35	translational diffusion coefficient
f/f_o	1.16	1.74 1.32	monomer tetramer	1.120	frictional ratio
$[h]_{c=o}$	3.5	10-15 5.7	monomer tetramer	3.35	ml · g^{-1}
$\beta^{\ddagger} \cdot 10^{-6}$	2.27	2.53 2.28	monomer tetramer	2.13	
$\bar{v}$	0.710	0.706		0.695	partial specific volume
$p = \frac{a}{b}$	3	14 6	monomer tetramer	3	axial ratio
$\nu = \frac{[h]}{\bar{v}}$	4.85	21.24 7.10	monomer tetramer	4.82	viscosity increment, Simha-factor

‡ - according to Mandelkern-Scheraga, using the equation

$$\beta = \frac{N \times S_{20,w} \cdot [h]^{1/3} \cdot h_s}{M_w^{2/3}(1-\bar{v}\rho)}$$, with h_s = viscosity of the solvent

1) in buffer A

2) in buffer B, containing 4M guanidinium-hydrochloride.

Simha-Einstein relation only is valid if the partial specific volume and the intrinsic viscosity represent the volume of 1 g protein, and that the hydrodynamic equivalent has almost the same volume, the asymmetry of phospholipase A is due to the extended polypeptide chain rather than to hydratation.

Considering the Mandelkern-Scheraga parameter ß [12] for phospholipase A, only an extended ellipsoid of revolution is consistent with the value obtained, while a value of 2.15×10^{-6} does not exceed that for oblate ellipsoids of any axial ratio.

Scattering curves were recorded in buffers A and B and care was taken to measure the different protein concentrations (crotoxin, crotapotin and phospholipase A) precisely under the same conditions in order to avoid aggregation. In the case of crotoxin a concentration dependence of the scattering curves were observed, e.g., by raising the magnesium concentration to 0.05 M and decreasing the potassium concentration from 0.06 M to 0.01 M. From sedimentation equilibrium experiments we detected a reversible dissociation⇌association equilibrium of crotoxin with an apparent association constant of $K_{app} = 1.2 \times 10^{9}$ (liter Mol^{-1}) and $\Delta G = -1.96$ (Kcal:Mol $Dimer^{-1}$). Except for phospholipase A, no further states of aggregation in case of crotapotin and crotoxin could be detected by means of low angle X-ray scattering in solution. Only for phospholipase A (see Tables 1 and 2) the radius of gyration and the other hydrodynamic parameters of the monomer and the tetramer are compatible with the feature of an extended shape with a high axial ratio. The molecular weights of the protein components listed in table 2, determined by small angle X-ray scattering, are about 5 % higher than that measured by sedimentation equilibrium. The degree of swelling q = 1.06, equivalent of a degree of hydration of 0.30 g H_2O per 1 g protein, reveals undoubtedly for phospholipase A that the high radius of gyration and the actual frictional ratio are due to the asymmetry of the monomer (tetramer), whereas the asymmetry of crotapotin is due to the influence of water on the polypeptide chain resulting in an oblate ellipsoid of revolution.

Determinations of the intrinsic viscosity of the crotoxin components reveal only for phospholipase A a high value of $[h] = 15$ for the monomer, whereas crotapotin and crotoxin indicate values for a more spherical particle with deviations from a sphere due to hydration or binding of cations.

Table 2.

Hydrodynamic properties of the crotoxin complex and its constituents, obtained by small angle X-ray scattering.

	crotoxin	phospholipase A (monomer)	crotapotin	
R_g (Å)	19.7	32.4	13.4	radius of gyration
R_o (Å)	25.7	36.0	17.5	Stokes' radius
^{+}V $(Å)^3$ $(x\ 10^{-3})$	25.5	14.2	13.5	Hydro-dynamic volume
$\bar{w}$	0.25 g H_2O/1 g	0.38 H_2O/1 g	0.48 H_2O/1 g	degree of hydrata-tion
MW	31,500	15,500	12,800	molecular weight
Dimensions	prolate ellipsoid 2a = 24.4 Å 2b = 12.0 Å	cylinder of L = 150.0 Å and D = 11.0 Å	oblate ellipsoid with 2b = 24.0 Å 2a = 10-12 Å	assuming different conforma-tions

$^{+}$ - assuming different shapes, for crotoxin a prolate ellipsoid with 2a = 24.4 Å, 2b = 12.0 Å, for crotapotin an oblate ellipsoid with 2a = 24.4 Å and 2b = 12.0 Å, and for phospholipase A an asymmetric cylinder with L = 150.0 Å and D = 11.0 Å.

DISCUSSION

From sedimentation, diffusion and viscosity measurements the crotoxin-complex was found to behave physically as an almost spherical particle with a Stokes' radius of R_o = 25.7 Å. The best hydrodynamic description is given by a prolate ellipsoid of revolution with p = a/b = 3 and cell dimensions of 2a = 24.4 Å, 2b = 12.0 Å, taking the measured degree of hydration

of 0.25 g H_2O/1 g protein into consideration. The components of the crotoxin complex, crotapotin and phospholipase A, are more asymmetric than crotoxin, where phospholipase A has a very high axial ratio. Crotapotin ist best described as being shaped like a hydrated sphere with a Stokes' radius of R_o = 17.5 Å at pH 7.5 (buffer B), but it behaves rather like an elongated molecule at pH 5.5 (buffer A) with an axial ratio of 1:6 and cell dimensions of 2b = 24.0 Å and 2b = 10-12 Å, assuming an oblate ellipsoid of revolution. In contrast to phospholipase A this asymmetry is due to hydration rather than to the polypeptide chain. From table 2 it can be seen that the calculated and measured radius of gyration for crotapotin shows conclusively that this molecule is described best in its shape as an oblate ellipsoid of revolution with an axial ratio of four and a radius of gyration of R_g = 13.4 Å, taking a degree of hydration of 0.4 g H_2O/1 g protein into account.

Moreover, the high axial ratio and the rather moderate degree of hydration of phospholipase A in comparison with crotoxin and crotapotin suggest that the high actual frictional coefficient f/f_o is not due to hydration but more to its anisometric (nonspherical) shape. Table 3 shows conclusively that the monomer and the tetramer can be described as a rod with cell dimensions for the monomer of L = 140-150 Å and D = 11.0 Å, and for the tetramer of L = 140-150 Å and D = 20-24 Å, respectively. Taking into consideration that the values in tables 1 and 2 are maximal values, corresponding to no degree of hydration, it would leave the radius of gyration practically unchanged because the hydrodynamic volume V_h has to be revised on the basis of the partial specific volume due to binding of some water, or even cations. Therefore, it is easy to discriminate between a random coil and other configurations, as listed in table 3.

In the present report the main findings are that the crotoxin complex consists of two molecules of different shape, one highly asymmetric and the other one an oblate ellipsoid of revolution with an axial ratio of 1:4. In case of reconstitution of the components phospholipase A and crotapotin, a particle of a sedimentation coefficient of 3S is formed by liberation of water from lysine and arginine residues of phospholipase A

Table 3.

Structural parameters of phospholipase A assuming different conformations (MW 15,400).

	R_g (Å)	R_e (Å)
Assuming random coil		
from $S_{20,w}$; $\nu = 0.665$	15.0	16.8
$D_{20,w}$; $\nu = 0.8875$	16.5	17.5
$[\eta]_{c=o}$;	16.3	
Assuming rigid rod		
from $S_{20,w}$, $D_{20,w}$, $[\eta]_{c=o}$	33.0	37.0
Experimental value (from X-ray scattering in solution)	32.4	36.0+
Calculated value, assuming a cylinder of L = 150 Å and D = 11.0 Å	32.8	35.9

- according to Kirkwood et al. [14]

+ - from Sephadex G 75 column chromatography.

[13]. Furthermore, by folding of the phospholipase A chain around or along the crotapotin molecule, the liberation of water is accompanied by a dissociation of $[H^+]$ ions from phospholipase A resulting in a decrease in the positive charge of phospholipase A by establishing hydrogen bonds between acidic, titritable carboxo-groups of crotapotin around pH 6.0 [13].

Acknowledgement: The authors wish to thank Dr. O. Westphal, Max-Planck-Institut für Immunbiologie, Freiburg, BRD, for critical reading and discussions of the manuscript.

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