Vol. 99, No. 1, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 16, 1981 Pages 292-301

CYTOCHROME b-561 OF THE BOVINE ADRENAL CHROMAFFIN GRANULES. MOLECULAR WEIGHT AND HYDRODYNAMIC PROPERTIES IN MICELLAR SOLUTIONS OF TRITON X-100

Torgeir Flatmark and Martin Grønberg
Department of Biochemistry, University of Bergen,
Årstadveien 19, N-5000 Bergen (Norway)

Received January 23, 1981

Summary - Cytochrome b-561 from the chromaffin granules, purified to homogeneity with a peptide mol.wt. of 20 500, revealed a marked tendency to aggregate in the absence of detergent. In micellar solutions of Triton X-100 it appeared as a single component on size-exclusion liquid chromatography, sedimentation equilibrium and sedimentation velocity analyses whether a purified or a crude preparation was analyzed. The binding of Triton X-100 was estimated to 44 mol/mol of protein or 1.36 g/g of protein. Based on hydrodynamic parameters, determined by the analytical ultracentrifuge, a mol.wt. of 53 300 was estimated for the hydrated protein.detergent complex, supporting a model in which one monomer of cytochrome b-561 is bound to 45 - 50 molecules of Triton X-100 in a mixed micelle.

The chromaffin granule membrane of bovine adrenal medulla contains a single heme protein, i.e. a b-type cytochrome (cytochrome b-561) (1,2), which has been isolated in a highly purified form by a non-ionic detergent procedure (3). Like other mammalian b-type cytochromes it contains a non-covalently bound iron protoporphyrin IX as the prosthetic group (3), and its high oxidation-reduction potential ($E_{m,7}$ = + 120 mV) (4) suggests a rather closed crevice structure (5). Our earlier work (3) revealed that the cytochrome behaves as a typical amphiphilic integral membrane protein, but some uncertainties were left with respect to the molecular weight of its monomeric form and state of aggregation when solubilized by Triton X-100. In the present paper, experiments

0006-291X/81/050292-10$01.00/0

are reported giving more precise values for the molecular weight and hydrodynamic properties of the cytochrome in micellar solutions of Triton X-100.

Materials and Methods

Highly purified chromaffin granule 'ghosts' were prepared from freshly collected bovine adrenal glands (6), and resuspended in 2 ml of K-phosphate buffer (50 mM) at pH 6.8, containing Triton X-100 (2 %,w/v) (3,7). The dissolved membranes were diluted and dialyzed against K-phosphate buffer (50 mM) at pH 6.8, KCl (100 mM) and Triton X-100 (0.1 %,w/v) at 4 °C, and centrifuged for 2 h at 10^5 x g at 4 °C; the supernatant contained approx. 5 mg protein/ml and 7.5 nmol cytochrome b-561/mg protein.

Cytochrome b-561 was purified essentially as described (3). On polyacryamide-gel electrophoresis in dodecyl sulfate, the purified protein revealed a single band (Fig. 1).

The Stokes' radius (R_s) was determined at 20 °C by high performance size-exclusion liquid chromatography on a G 3000 SW column (60 cm x 7.5 mm, prepacked from Toyo Soda, Manufacturing Co., Ltd.,Japan) equilibrated and eluted with K-phosphate buffer (50 mM) at pH 6.8, containing KCl (0.1 M) and in certain experiments also Triton X-100 (0.1 %,w/v); the flow rate was 1 ml/min. Cytochrome b-561 was detected using a variable wavelength detector (SpectroMonitor 1200 from Laboratory Data Control,FL, U.S.A.) set at 415 nm (isosbestic point for the reduced and oxidized form (3)). Stokes' radius was estimated as described (8) or from results obtained by sedimentation equilibrium and velocity analyses (9)(see below).

Sedimentation velocity and equilibrium experiments were performed at 20 °C in an MSE Centriscan 75 analytical ultracentrifuge. The absorbance at 418 nm in the cell was measured during centrifugation using the UV lamp and an interference filter with maximum light transmission at 418 nm.

The amount of Triton X-100 bound to cytochrome b-561 was calculated from the equation (10),

$$M_p(1 - \phi'\rho) = M_p(1 - \bar{v}_p\rho) + \bar{v}\, M_D(1 - \bar{v}_D\rho) \qquad (1)$$

where the buoyant density factor $M_p(1 - \phi'\rho)$ (9,11) was determined at 20 °C from sedimentation equilibrium analyses using the equation (9),

$$M_p(1 - \phi'\rho) = (2\,RT/\omega^2)(2.303\; d \log c/d\, r^2) \qquad (2)$$

where M_p is the molecular weight of protein in the sedimenting particle (exclusive of hydration or bound detergent) and ϕ' is the effective partial specific volume (9).

Triton X-100 binding was also estimated by high performance size-exclusion liquid chromatography using the same solvent as described above containing ^{3}H-labelled Triton X-100 (generously supplied by Dr. Alan M. Rothman, Rhom and Haas

Co.,PA,U.S.A.). The specific radioactivity of the detergent was about 50 000 cpm/mg Triton X-100.

Polyacrylamide-gel electrophoresis in dodecyl sulfate (12) was performed using gels containing acryalmide (6 %,w/v) and N,N'-methylene-bisacryalmide (0.16 %,w/v). The purified cytochrome was dissolved in Na-phosphate buffer (10 mM) at pH 7.1, containing dodecyl sulfate (1 %,w/v) and β-mercaptoethanol (1 %,v/v) and dialyzed against this buffer containing dodecyl sulfate (0.1 %,w/v) and β-mercaptoethanol (0.1 %,v/v). The electrophoresis calibration kit of Pharmacia, Uppsala, Sweden was used for molecular weight determination.

Difference spectra were measured in a Cary Model 219 recording spectrophotometer. The concentration of cytochrome b-561 in the 'ghost' preparations were determined by an Aminco-Chance dual wavelength spectrophotometer (2).

Protein in granule 'ghosts' was determined by the Comassie Brilliant Blue method of Bradford (13) and purified protein by the micro biuret method of Goa (14) using bovine serum albumin as a standard.

Results

Electrophoresis on polyacrylamide gel

When purified cytochrome b-561 was subjected to dodecyl sulfate gel electrophoresis, a single band was observed (Fig. 1A); the mol.wt. was estimated to approx. 22 000 based on the mobility relative to standard hydrophilic proteins (Fig. 1B). Based on previous amino acid analyses (3), a peptide mol.wt. of 20 500 was estimated (Table I).

High performance size-exclusion liquid chromatography

Purified cytochrome b-561 was eluted with the void volume (t_R = 8.28 min, n = 3) on high performance size-exclusion liquid chromatography (Fig. 2A) using a solvent with no detergent added. However, when the solvent contained a micellar concentration of Triton X-100 (0.1 %,w/v), a single component was eluted at t_R = 12.52 min (K_{av} = 0.23, n = 5) (Fig. 2B). Based on a comparison with standard hydrophilic globular proteins, a Stokes' radius of 34.7 Å was estimated for the cytochrome·Triton complex (Table I).

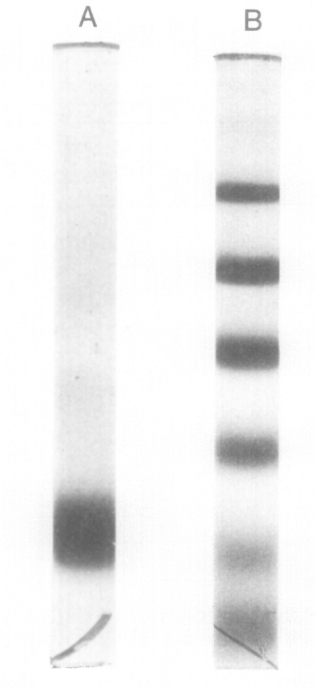

Fig. 1. A. Polyacrylamide-gel electrophoresis in sodium dodecyl sulfate of purified cytochrome b-561. Approx. 30 µg of protein was applied to the gel. B. Standard hydrophilic proteins of mol.wt. (from top) : 94 000, 67 000, 43 000, 30 000, 20 100 and 14 400.

When a crude preparation of the cytochrome (Triton X-100 solubilized chromaffin granule ghosts) was applied to the column, three components were observed. Two minor components (not identified) were eluted with the void and total volume, respectively,and the major component was identified as cytochrome b-561 (t_R = 12.31 min and K_{av} = 0.22, n = 3) (Fig. 2C) with a Stokes' radius of 35.5 Å (Table I).

Estimation of Triton binding by sedimentation equilibrium analyses

A typical sedimentation equilibrium experiment is shown in Fig. 3A and B. From the linear plot of log A vs r^2 the buoyant density factor of the cytochrome·Triton complex was

Table I

Hydrodynamic properties of the cytochrome b-561·Triton X-100 complex[a]

	Purified cytochrome b-561	Crude cytochrome b-561
s_o (S)[b]	-	2.31
$M_p(1 - \phi'\rho)$[c]	-	7896.0
R_s (Å)	-	35.6[d]
	34.7[e]	35.5[e]
$\bar{\nu}$ (mol/mol)[f]	-	43.7
δ_D (g/g)[g]	-	1.36
$\bar{v}_p$ (cm^3/g)[h]	0.73(4)	-
$\bar{v}_c$ (cm^3/g)[i]	0.82(2)	0.82(2)
M_c [j]	53 300	53 300
M_p [k]	20 500	-

[a] The solvent was K-phosphate buffer (50 mM) at pH 6.8, KCl (100 mM) and Triton X-100 (0.1 %, w/v); 20 °C.

[b] Sedimentation coefficient determined at infinite dilution (see Fig. 3D).

[c] Buoyant density factor (9,11) determined by sedimentation equilibrium centrifugation (Fig. 3B).

[d] Estimated according to ref. 9 using the values for s and the buoyant density factor determined under identical conditions (see text).

[e] Based on high performance size-exclusion liquid chromatography (see text).

[f] Average number of mol detergent bound per mol of protein by assuming a monomeric state of the protein in the complex.

[g] Amount of detergent bound per g of protein based on a monomeric mol.wt. of M_p = 20 500 (see footnote k) below).

[h] Based on the amino acid composition (ref. 3).

[i] Partial specific volume of the protein·detergent complex estimated according to ref. 9; $\bar{v}$ = 0.908 cm^3/g was assumed for Triton X-100 (10).

[j] Molecular weight of the cytochrome b-561·Triton complex estimated according to ref. 9 using the hydrodynamic parameters s_o = 2.31 S, R_s = 35.5 Å and $\bar{v}_c$ = 0.822 cm^3/g.

[k] Molecular weight of peptide in the protein·detergent complex estimated from the amino acid analyses (ref. 3).

estimated (from eqn. (2)) to be $M_p(1 - \phi'\rho)$ = 7896. This value is too small to be interpreted in terms of an aggregated species of the cytochrome, i.e. $M_p(1 - \phi'\rho)$ was greater than 20 500$(1 - \bar{v}_p\rho)$ = 5375, but less than 41 000$(1 - \bar{v}_p\rho)$ =

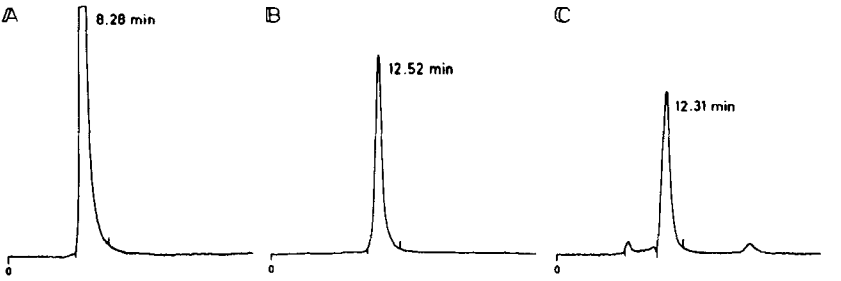

Fig. 2. High performance size-exclusion liquid chromatography of 21.9 µg (A) and 4.1 µg (B) purified cytochrome b-561 and 5.5 µg of a crude cytochrome preparation (Triton X-100 extract of chromaffin granule membranes) (C). The solvent consisted of K-phosphate buffer (50 mM) at pH 6.8 and KCl (0.1 M) with no (A) or a micellar concentration of Triton X-100 (0.1 %,w/v) (B and C). 20 µl samples were injected into the liquid chromatograph. The variable wavelength detector was set at 415 nm and A (cm^{-1}) = 0.02 at full scale; the attenuation of the recording integrator was set at 8. For experimental details, see text. The retention times represent average values of 3 to 5 experiments.

10 750 (for estimation of M_p, see Table I). The calculation of detergent binding was thus based on a monomeric state of the protein. The average number of mol detergent bound per mol of protein ($\bar{\nu}$) was estimated (from eqn. (1)) to be 43.7 giving a value of δ_D = 1.36 g Triton/g of protein.

Estimation of Triton binding by high performance size-exclusion liquid chromatography

When the G 3000 SW column was equilibrated with ^3H-labelled Triton X-100, a peak of radioactivity co-eluted with the peak of purified cytochrome b-561 (figure not shown), immediately followed by a trough in the baseline radioactivity. From such experiments it was estimated that the purified cytochrome b-561 binds approx. 1.3 g Triton X-100/g of protein.

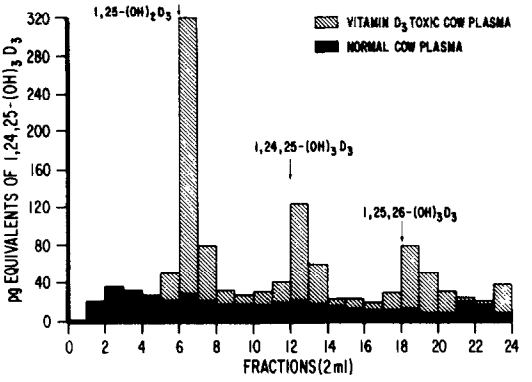

Figure 1. HPLC receptor binding profile of plasma extracts from normal and vitamin D_3-treated cows after purification on Sephadex LH-20. The profile represents elution from a Zorbax Sil column developed in hexane:isopropanol (83:17). Arrows indicate the elution positions of authentic standards.

respective synthetic standards on the Zorbax ODS column (data not shown). The comigration of the receptor binding activities with synthetic 1,25(S),26-$(OH)_3D_3$ on HPLC columns with different selectivities suggested that this new vitamin D_3 metabolite was 1,25,26-$(OH)_3D_3$.

Five hundred ng of this new metabolite of vitamin D_3 were isolated from 16 l of plasma from vitamin D_3-treated cows and subjected in part to mass spectrometric analysis. The mass spectrum of this new metabolite is characteristic of a trihydroxylated vitamin D_3 metabolite (Fig. 3). The molecular ion at m/e 432 and the three sequential losses of H_2O at m/e 414, 396, and 378 indicate the presence of 3 hydroxyl groups in addition to the 3β-hydroxyl group. The peak at m/e 269 arises from side-chain cleavage and loss of H_2O from the remaining fragment. Loss of a second H_2O molecule provides the peak at m/e 251. The peak at m/e 152 arises from formal cleavage between carbons 7 and 8 to give rise to the A ring, plus carbons 6 and 7 fragment, containing 2 hydroxyl groups. Loss of H_2O from m/e 152 provides the base peak at m/e 134. The fragments at m/e 152 and 134 illustrate that the secosteroid nucleus of the vitamin has remained unchanged.

estimated to s_o = 2.31 S (Fig. 3D). The Stokes' radius of the cytochrome·detergent complex was estimated (ref. 9) to R_s = 35.6 Å using the s value and buoyant density factor determined under identical conditions (Fig. 3B and D). This value is in close agreement with that obtained by size-exclusion chromatography (Table I). From the hydrodynamic parameters s_o = 2.31 S, R_s = 35.5 Å and $\bar{v}_c$ = 0.822 cm^3/g, an approximate mol.wt. of 53 300 was estimated for the hydrated cytochrome· Triton complex (Table I).

Discussion

Previous physico-chemical studies on cytochrome b-561, the integral heme protein of the chromaffin granule membrane, have given different values for its molecular weight, depending on the analytical method used (1). Two of the problems faced with in these studies were the strong tendency of the cytochrome to aggregate in the absence of added detergent, and the complete dissociation of the prosthetic group (protoheme IX) in the presence of ionic detergents (e.g. dodecyl sulfate) (1).

The present study has confirmed our previous finding (3) that purified cytochrome b-561 aggregates in the absence of added non-ionic detergent (Fig. 2A). On the other hand, in micellar solutions of Triton X-100 the cytochrome appeared as a single component on high performance size-exclusion liquid chromatography (Fig. 2B) as well as on sedimentation equilibrium (Fig. 3A and B) and sedimentation velocity (Fig. 3C and D) measurements. Within the experimental error, the same Stokes' radius was estimated for the cytochrome whether a crude or a highly purified preparation of the protein was analyzed (Table I), indicating an identical size for the

hydrated cytochrome·Triton complex at the two experimental conditions. This conclusion was confirmed by analytical ultracentrifugation in which both the crude (Fig. 3C) and the highly purified cytochrome (data not shown) sedimented with homogeneous boundaries and similar s values. The approximate molecular weight of the hydrated cytochrome b-561·Triton complex was calculated to be 53 300 based on the hydrodynamic parameters given in Table I. This particle weight is compatible with a model in which one cytochrome monomer is associated with 40 - 50 molecules of detergent (mol.wt. 640). The amount of detergent bound indicates that the binding can be explained by a co-micellation of the monomeric form of the cytochrome with the detergent. However, the aggregation number of Triton in the complex is approx. half of that observed in pure detergent micelles, estimated to be about 100 (15,16). Thus, this amphiphilic protein, in contrast to microsomal cytochrome b_5 (9), seems to perturb the normal micelle aggregation of Triton X-100 monomers sufficient to alter the micelles that are formed.

The same Stokes' radius and s value was estimated for the cytochrome b-561·Triton complex whether a crude or a highly purified preparation of the protein was used. This finding indicates that if the cytochrome interacts with other integral proteins of the granule membrane, this interaction is weak and is completely abolished by the dissolution of the membrane by Triton X-100 at 0 °C.

Acknowledgements. This work was supported by the Norwegian Council on Cardiovascular Research and the Nordic Insulin Foundation. M.G. is a fellow of the Norwegian Research Council for Science and the Humanities. The valuable assistance of Dr. Dag Helland in the sedimentation experiments is greatly appreciated.

References

1. Flatmark,T.,Terland,O. and Helle,K.B. (1971) Biochim. Biophys. Acta 226, 9-19.
2. Terland,O., Silsand,T. and Flatmark,T. (1974) Biochim. Biophys. Acta 359, 253-256.
3. Silsand,T. and Flatmark,T. (1974) Biochim. Biophys. Acta 359, 257-266.
4. Flatmark,T. and Terland,O. (1971) Biochim. Biophys. Acta 253, 487-491.
5. Kassner,R.J. (1973) J. Amer. Chem. Soc. 95, 2674-2677.
6. Terland,O. and Flatmark,T. (1980) Biochim. Biophys. Acta 597, 318-330.
7. Slinde,E. and Flatmark,T. (1976) Biochim. Biophys. Acta 455, 796-805.
8. Laurent,T.C. and Killander,J. (1964) J. Chromatog. 14, 317-330.
9. Tanford,C., Nozaki,Y., Reynolds,J.A. and Makino,S. (1974) Biochemistry 13,2369-2376.
10. Robinson,N.C. and Tanford,C. (1975) Biochemistry 14,369-378.
11. Hersh,R.T. and Schachman,H.K. (1958) Virology 6,234-
12. Weber,K. and Osborne,M. (1969) J. Biol. Chem. 244, 4406-4412.
13. Bradford,M.M. (1976) Anal. Biochem. 72, 248-254.
14. Goa,J. (1953) Scand. J. Clin. Lab. Invest. 5, 218-222.
15. Becker,P. (1967) in Nonionic Surfactants (Schick,M.J.,ed.), Marcel Dekker, New York, p. 495.
16. Lewis,M.S. and Kirk,W.D. (1974) Fed. Proc. 33,1230.