Dissociation of CNPase dimer into catalytically active monomers by 1,4-dithiothreitol and urea

Protein-promoted reassembly

H.W. Müller

Friedrich-Miescher-Laboratorium der Max-Planck-Gesellschaft, Spemannstr. 37–39, 7400 Tübingen, FRG

Received 1 June 1982

CNPase *Monomer – dimer relationship* *Intersubunit disulfide bridges* *Central nervous system* *2',3'-cyclic nucleotides* *Myelin*

1. INTRODUCTION

Membrane-bound 2',3'-cyclic nucleotide 3'-phosphodiesterase activity (CNPase, EC 3.1.4.37) is most prominent in the mammalian central nervous system but low levels of CNPase activity have also been detected in other mammalian tissues as well as in bacteria (review [1]). A great variety of estimations has been reported for bovine brain CNPase, 30 000–100 000 M_r [2–8]. This diversity in M_r has been investigated in detail by limited proteolytic digestion, plant lectin affinity chromatography and immunochemical analysis [9] using purified bovine brain CNPase isolated as in [10]. Under reducing conditions, the enzyme resolves into a polypeptide doublet (M_r 51 000–54 000) in SDS–polyacrylamide gels [1]. Two catalytically active molecular forms of CNPase were identified: a low-M_r form of the enzyme ($M_r \sim 50\,000$) and a high-M_r form ($M_r \sim 100\,000$) providing evidence for a monomer–dimer relationship in CNPase [1].

These experiments support our hypothesis as they demonstrate the presence of covalent and non-covalent interactions between the subunits in the high-M_r form of CNPase. Experiments are described in which were observed:

(i) Nearly complete dissociation of purified CNPase dimer into catalytically active monomers; and

(ii) Reversible interconversion of the monomeric CNPase into a high-M_r enzyme form.

Furthermore, the specific activities of both CNPase forms are compared and the molecular form of the enzyme possibly occurring in vivo is discussed.

Direct correspondence to present address: Department of Neurobiology, Stanford University School of Medicine, Stanford, CA 94305, USA

2. MATERIALS AND METHODS

2',3'-Cyclic nicotinamide–adenine dinucleotide phosphate (2',3'-cNADP), bovine serum albumin (BSA), carbonic anhydrase and 1,4-dithiothreitol (DTT) were purchased from Sigma Chemical Co. (St Louis MO). Urea was produced by Bio-Rad Lab. (Richmond CA). Human γ-globulin was obtained from Miles Lab. (Elkhart IL) and all other chemicals used were of analytical grade purchased from Merck (Darmstadt).

Rapid isolation of CNPase from bovine brain white matter was performed under non-reducing conditions by guanidinium–HCl extraction and carboxymethyl–Sephadex column chromatography as in [10].

CNPase activity was estimated using 2',3'-cNADP as substrate in the combined enzyme assay [11]. One unit (U) of CNPase is defined as the amount of enzyme that produces 1 μmol product (2'-NADP)/min at 37°C in 50 mM sodium acetate buffer (pH 5.5). Protein was measured as in [12].

Sedimentation rate analyses were carried out by centrifugation of CNPase in linear 32 ml sucrose density gradients (10–35%, w/v). All gradients were prepared in 50 mM 2-(*N*-morpholino)–ethane sulphonic acid–NaOH (MES–NaOH) buffer (pH 6.4) containing 1 mM EDTA and centrifuged at

Published by Elsevier Biomedical Press
00145793/82/0000–0000/$2.75

81 500 × g_{av} for 43 h in a Beckman SW27 rotor. Some sucrose gradients contained 1 mM DTT. Fractions of 1 ml were collected. Molecular mass estimations were based on co-centrifugation of protein standards: human γ-globulin (M_r 150 000), BSA (M_r 68 000) and carbonic anhydrase (M_r 29 000).

3. RESULTS AND DISCUSSION

Sedimentation rate analyses of isolated bovine brain CNPase (spec. act. 700 U/mg protein) were performed in linear sucrose density gradients under both non-reducing and reducing conditions. Fig.1a represents the activity profile of purified CNPase in a sucrose gradient centrifuged under non-reducing conditions. The peak activity corresponds to app. M_r 100 000 (100 kDa). An additional but minor shoulder of activity appears at > 150 000 M_r. The 100 kDa CNPase activity, as indicated by (D), has recently been identified by gel filtration as the high-M_r enzyme form being composed of two polypeptide chains (M_r 51 000–54 000) [1]. However, in the presence of a large molar excess of the reducing agent DTT (1 mM) two peaks of CNPase activity appeared in the sucrose gradient (fig.1b), corresponding to the M_r-values of 100 kDa (D) and 50 kDa (M), respectively. This indicates that the enzyme activity obtained in a single peak at 100 kDa under non-reducing conditions (fig.1a) is partially dissociated by the thiol reagent and shifted towards a lower M_r (50 000). At 1 mM, DTT has no effect on the catalytical activity of CNPase since the total activities remained almost unchanged in both gradients (fig.1a,b). This result further implies that the specific activities of both enzyme forms were roughly identical as the same amount of enzyme (30 μg protein) was applied on both sucrose gradients. The ~50 000 M_r estimated for the CNPase activity peak (M) after partial dissociation of the high-M_r form of the enzyme corresponds with the low-M_r form in [1] and agrees with the M_r-values of the polypeptide subunits of brain CNPase estimated in SDS–polyacrylamide gels under reducing conditions [1]. These results indicate that the 50 kDa CNPase form represents a catalytically active monomer (M), consisting of a single polypeptide chain, whereas the 100 kDa enzyme form represents a dimer (D).

Nearly complete dissociation of CNPase dimer

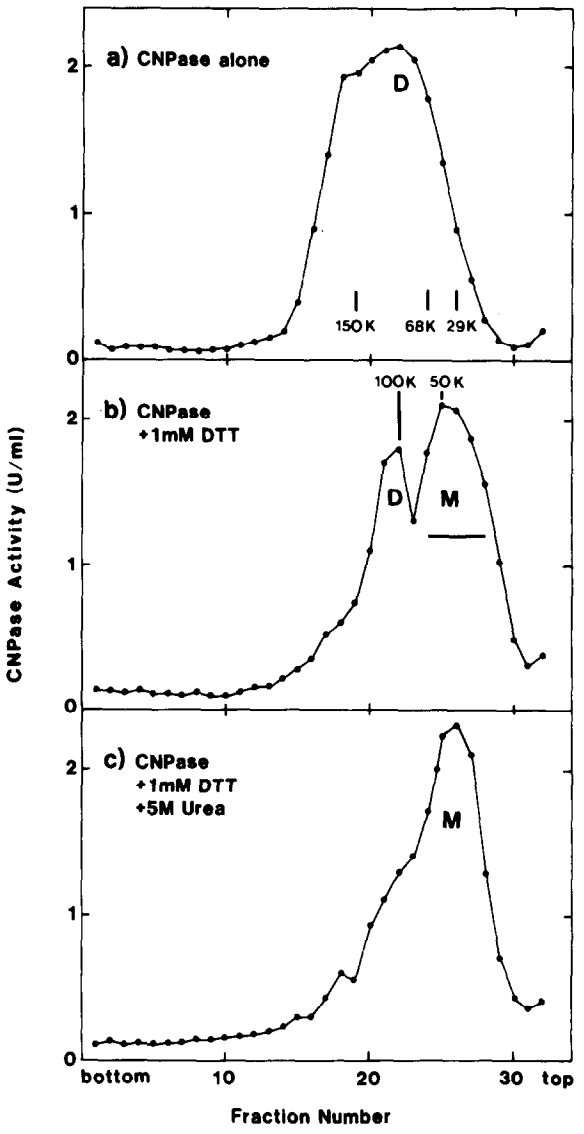

Fig.1. Activity profiles of bovine brain CNPase after sucrose density gradient centrifugation: (a) under non-reducing conditions; (b) in the presence of 1 mM DDT; and (c) in the presence of 1 mM DTT after preincubation of the enzyme protein in 1 mM DDT and 5 M urea for 30 min and subsequent dialysis. (D) and (M) indicate dimeric and monomeric enzyme forms, respectively. Sample volumes of 1 ml containing 30 μg enzyme protein (spec. act. 700 U/mg) were applied on each gradient. The M_r-marker proteins (human γ-globulin, BSA and carbonic anhydrase) were run in parallel gradient tubes and the positions of their peak maxima are indicated in (a). Further details are given in section 2.

into monomers (M) occurred upon incubation of purified CNPase in the presence of both DTT (1 mM) and urea (5 M) at 25°C for 30 min prior to dialysis to remove urea and sucrose gradient centrifugation (fig.1c). Urea at 5 M totally inhibits CNPase activity. However, the inhibition is reversible and the enzyme activity can be restored by dialysis so that there is no substantial change in enzyme activity (see fig.1c) when compared to the gradients in fig.1a and 1b. Urea alone is unable to dissociate the CNPase subunits (not shown).

From [1] a reaggregation of CNPase monomers (50 kDa) to form the 100-kDa enzyme complex would be predicted. To investigate the reversibility of the interconversion of both enzyme forms, 5 gradient fractions were pooled from the activity peak (M) in fig.1b as indicated by the bar (total vol. 5 ml, 10 units of CNPase activity). DTT and sucrose were removed by dialysis (4°C, 15 h) against 2 × 500 ml 50 mM MES–NaOH buffer (pH 6.4) containing 1 mM EDTA. The dialyzed enzyme sample was then divided into two halves and each fraction was rerun on a separate sucrose gradient either in the absence or in the presence of BSA. When BSA was omitted the main peak (M) of CNPase activity appeared at the same position (50 kDa) in the sucrose gradient (fig.2a) as compared to the original fractions (M) in fig.1b, indicating that CNPase

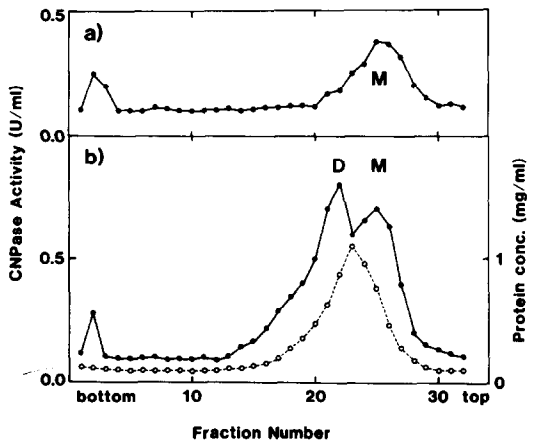

Fig.2. Sucrose density gradient centrifugation of monomeric CNPase: (a) in the absence of additional protein; or (b) in the presence of 10 mg BSA/ml; (•—•) CNPase activity; (○—○) protein concentration. Further details are described in sections 2 and 3.

monomers do not spontaneously reassemble into a dimer after removal of the reducing agent (air-oxidation). However, in the presence of 10 mg BSA/ml added to the enzyme sample prior to centrifugation, two main peaks of CNPase activity were resolved in the gradient (fig.2b). The peak activities correspond to ~ 100 000 M_r (D) and 50 000 M_r (M) showing the reappearance of the high-M_r enzyme form upon partial reaggregation of monomers. The latter result corroborates our finding that various proteins, among which is found BSA, may cause the (partial) conversion of the 50-kDa CNPase form into the 100-kDa enzyme [1].

The CNPase activity recovered in the gradient of fig.2a represented only ~ 50% (2.5 U) of the expected activity. This loss in enzyme activity might be due to precipitation indicated by the small peak of (residual) activity near the bottom of the gradient tube. However, the presence of BSA in the enzyme sample (fig.2b) caused not only the reappearance of the high-M_r CNPase form (D) but also stimulated the enzyme up to a total activity of 10 U. This value represents a 2-fold stimulation over the expected activity of 5 U but when compared with the corresponding enzyme fraction centrifuged in the absence of additional protein (fig.2a) the CNPase activity is stimulated 4-fold in the presence of BSA.

Bovine brain CNPase, isolated under non-reducing conditions, is a protein dimer made up of two 50-kDa polypeptide subunits. The dimeric structure of the enzyme is easily dissociated by DTT and urea suggesting the presence of intersubunit disulfide bridges and hydrogen bonds necessary for maintaining the quarternary structure. The monomeric enzyme form thus obtained is catalytically active and stable; however, the CNPase dimer seems to be the form of the enzyme found in vivo in brain. It cannot be ruled out that there may exist even larger complexes of the enzyme in this tissue (see also fig.1a) which were not isolated by the present preparation method which included guanidinium hydrochloride extraction. From the almost identical specific activities observed for both enzyme forms (fig.1a, 1c) it can further be concluded that the number of catalytic sites remained unchanged upon dissociation of the dimer into monomers.

Knowledge of the nature of the bonds involved in the stabilization of the quarternary structure of the CNPase dimer and the availability of an enzymati-

cally active monomeric enzyme also suggest that reversible interconversion of both enzyme moieties may be useful in the evaluation of its still unknown biological function in the central nervous system. In [1] we outlined common biochemical properties shared by CNPase and RNAase and suggested a possible function of CNPase in the RNA metabolism. A similar type of CNPase activity processing RNA in wheat germ has been described in [13].

ACKNOWLEDGEMENTS

The author gratefully acknowledges the technical assistance of stud. rer. nat. Carlos Pascual and the helpful suggestions and discussions of Dr Wilfried Seifert.

REFERENCES

[1] Müller, H.W., Clapshaw, P.A. and Seifert, W. (1981) FEBS Lett. 131, 37–40.
[2] Hugli, T.R., Bustin, M. and Moore, S. (1973) Brain Res. 58, 191–203.
[3] Guha, A. and Moore, S. (1975) Brain Res. 89, 279–286.
[4] Clapshaw, P.A. and Seifert, W. (1977) Hoppe-Seyler's Z. Physiol. Chem. 358, 1189.
[5] Drummond, R.J. (1979) J. Neurochem. 33, 1143–1150.
[6] Nishizawa, Y., Kurihara, T. and Takahashi, Y. (1980) Biochem. J. 191, 71–82.
[7] Sprinkle, T.J., Grimes, M.J. and Eller, A.G. (1980) J. Neurochem. 34, 880–887.
[8] Suda, H. and Tsukada, Y. (1980) J. Neurochem. 34, 941–949.
[9] Müller, H.W., Clapshaw, P.A. and Seifert, W. (1981) J. Neurochem. 36, 2004–2012.
[10] Clapshaw, P.A., Müller, H.W. and Seifert, W. (1981) J. Neurochem. 36, 1996–2003.
[11] Sogin, D.D. (1976) J. Neurochem. 27, 1333–1337.
[12] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[13] Konarska,.M., Filipowicz, W. and Gross, H.J. (1982) Proc. Natl. Acad. Sci. USA 79,1474–1478.