TWO MOLECULAR FORMS OF THE ISOLATED BRAIN ENZYME 2′,3′-CYCLIC NUCLEOTIDE 3′-PHOSPHODIESTERASE

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1. Introduction

The enzyme 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNPase, EC 3.1.4.37) hydrolyzes the 3′-phosphodiester bond of 2′,3′-cyclic phosphodiester nucleotides to produce the 2′-monophosphate nucleotides exclusively. The 2′,3′-cyclic nucleotides are known to occur as intermediates during enzymatic degradation of RNA. Evidence for this conclusion has been based on experiments carried out with purified RNAase preparations from various sources, in which 2′,3′-cyclic nucleotides, as well as terminal 2′,3′-cyclic phosphodiesters of oligonucleotides, are formed as products during short periods of RNAase action on RNA [1].

CNPase activity has been found in a variety of mammalian tissues and cells such as brain [2], spleen [3], pancreas [4], intestinal mucosa [2], as well as in fibroblasts [5], and red blood cells [6]. This enzyme activity has also been found in bacteria [7]. However, CNPase activity is most prominent in membranes of the central nervous system (CNS) [2] and shows a remarkably high activity in myelin [8], a tissue fraction in which only a few enzymes are present in readily measurable quantities. Developmental changes in CNPase activity have been shown to correlate well with myelination patterns in chick brain and spinal cord [9] and parts of rat brain [10]. Due to the close association of CNPase to myelin, CNS white matter has preferentially been used for CNPase preparations [11–13]. Several attempts have been made to purify and characterize brain CNPase. However, the molecular weights reported are rather inconsistent, ranging from 30 000 to 100 000 [11,13–17]. The molecular structure of this prominent brain enzyme, therefore, is still unclear.

In the present study we describe molecular weight determinations performed by three different methods using purified brain CNPase. Based on these analyses we have identified two active enzyme forms which provide strong evidence for a model proposed here of polypeptide composition of brain CNPase.

2. Materials and methods

2′,3′-Cyclic nicotinamide-adenine dinucleotide phosphate (2′,3′-cNADP), bovine serum albumin (BSA), and egg albumin were purchased from Sigma Chemical Co. (St Louis). 2′,3′-Cyclic nucleotide adenosine monophosphate (2′,3′-cAMP), 2′,3′-cGMP, 2′,3′-cUMP, and 2′,3′-cCMP were obtained from Boehringer (Mannheim). The tritium labelled nucleotides 8-[^{3}H]2′,3′-cAMP and 2′,3′-cGMP were synthesized by Amersham Buchler (Braunschweig). Acrylamide, bisacrylamide, ammonium persulfate, sodium dodecyl sulfate, Coomassie Brilliant Blue R-250, cytochrome *c*, and ferritin were obtained from Serva (Heidelberg). Human gamma globulin was produced by Miles Laboratories (Elkhart). All other chemicals used in these studies were of analytical grade purchased from Merck (Darmstadt).

Isolation of CNPase from bovine brain white matter was accomplished by guanidinium hydrochloride extraction and carboxymethyl Sephadex column chromatography using established procedures [12,18]. The specific activity of this preparation was 2500 U/mg protein, representing a 250-fold purification of the enzyme from white matter (specific activity approx. 10 U/mg protein).

CNPase activity was routinely assayed in 50 mM sodium acetate buffer, pH 5.5, containing 1 mg/ml BSA and 0.1% (v/v) NP40, by the conversion of 2′,3′-cyclic NADP to the 2′-phosphodiester (2′-NADP) according to the method of Sogin [19]. One unit of

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CNPase is defined as the amount of enzyme that produces 1 μmol of 2′-NADP per min at 37°C.

For the analysis of substrate specificity of CNPase, various 2′,3′-cyclic nucleoside monophosphates, as well as cNADP, were tested (cAMP, cGMP, cCMP, cUMP). The amount of conversion of substrate to product was estimated spectrophotometrically [11]. In the cases of 2′,3′-cAMP and 2′,3′-cGMP the activity was verified using radioactively labelled precursors [10], in which case substrate and product nucleotides were separated by thin layer chromatography.

Protein concentration was measured using a protein assay according to Bradford [20].

Sodium dodecyl sulfate (SDS) gel electrophoresis was carried out on calibrated 12% (w/v) polyacrylamide gels under reducing conditions according to Laemmli [21]. Prior to electrophoresis the protein samples were boiled for 2 min at 100°C in the presence of 2% (w/v) SDS and 5% (v/v) 2-mercaptoethanol. Protein bands were detected by staining of the gels with Coomassie Brilliant Blue, and the molecular weights were estimated using BSA (68 000), ferritin (50 000), carbonic anhydrase (29 000), and cytochrome *c* (12 300) as standards.

Gel filtration of CNPase was carried out at 4°C on a Sepharose 6B column (2.5 × 90 cm), equilibrated with 50 mM 2-(*N*-morpholino) ethane sulfonic acid-NaOH (MES–NaOH) buffer, pH 6.0, containing 150 mM NaCl, and 5% (v/v) glycerol. Human gamma globulin (150 000), BSA (68 000), egg albumin (45 000) and cytochrome *c* (12 000) were used as molecular weight standards.

Sedimentation rate analyses were performed by centrifugation of CNPase in linear 32 ml sucrose density gradients (10–35%, w/v). All gradients were prepared in 50 mM MES–NaOH buffer, pH 6.2, containing 1 mM of each EDTA and dithiothreitol and centrifuged for 15 h at 150 000 × g_{av} in a titanium vertical rotor using a Beckman ODT 2 ultracentrifuge. One-ml fractions were collected. Molecular weight estimations were based on co-centrifugation of the protein standards used in gel filtration except that egg albumin was replaced by carbonic anhydrase (29 000).

3. Results and discussion

In accordance with other studies on substrate specificity of CNPase [2,6] our preparation hydrolyzes the various 2′,3′-cyclic nucleotides in the following order (specific activities are given in brackets): cNADP (2500 U/mg) > cAMP (1450 U/mg) > cGMP (900 U/mg) > cCMP (320 U/mg) > cUMP (160 U/mg). The K_m values of our CNPase preparation have been determined to be $5.5 \cdot 10^{-4}$ M and $3.7 \cdot 10^{-4}$ M for cNADP and cAMP, respectively.

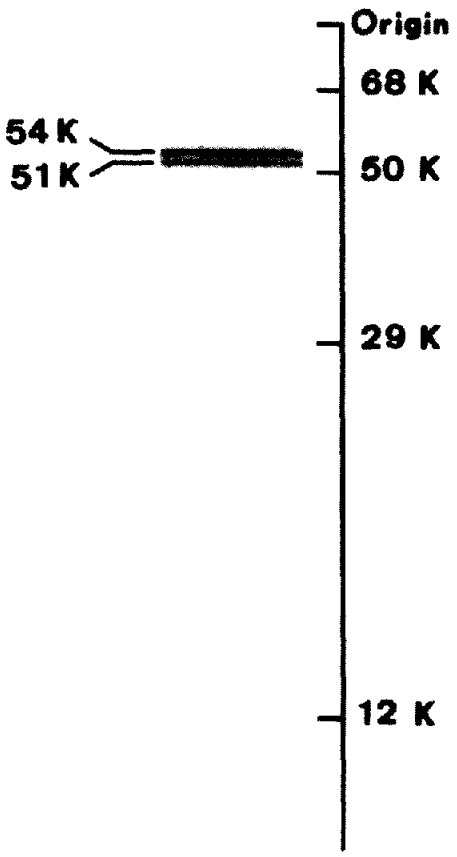

Fig.1. SDS-polyacrylamide gel electrophoresis of bovine brain CNPase. 40 μg of enzyme protein were prepared and run as described in section 2 (K = kilodalton).

In SDS-polyacrylamide gel electrophoresis (fig.1) freshly isolated CNPase migrated as a polypeptide double band with the apparent M_r values of 54 000 ± 1000 and 51 000 ± 1000, respectively. During storage of the enzyme at 4°C, low M_r breakdown products of the protein double band may appear (not shown).

When the molecular weight of CNPase was determined under native conditions using Sepharose 6B gel filtration (fig.2), the enzyme activity was eluted at a volume (210 ml) corresponding to M_r 120 000 ±

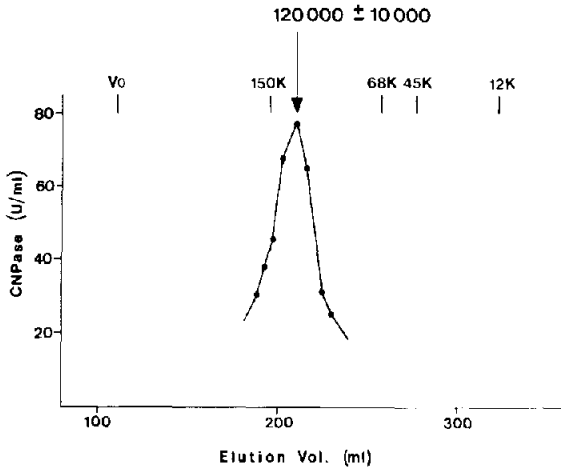

Fig.2. Elution profile of brain CNPase (1.0 mg protein) from Sepharose 6B column. The void volume (V_o) as well as the elution volume of marker proteins are indicated.

10 000 (high molecular weight enzyme form), agreeing rather well with a previous estimation of about 100 000 using a similar procedure [11].

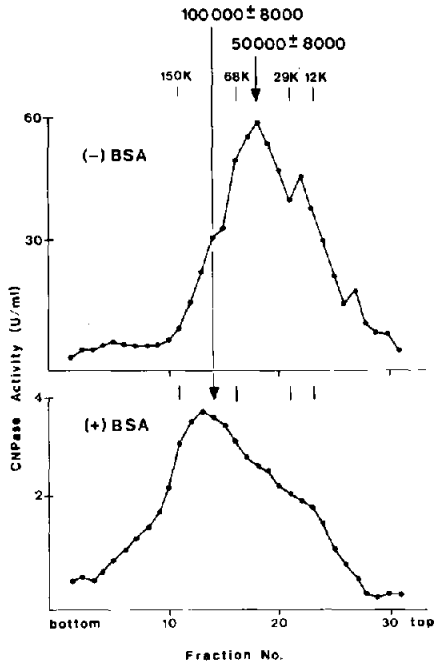

Fig.3. Sucrose density gradient centrifugation of brain CNPase in the absence of BSA (upper profile, 500 μg enzyme protein), or in the presence of BSA (lower profile, 50 μg enzyme protein). The position of marker protein peaks are indicated. Further details are given in section 2.

Sedimentation rate analyses of CNPase in linear sucrose density gradients were performed in the absence or presence of BSA (10 mg/ml) in the enzyme sample loaded on top of the gradient. When BSA was omitted the main peak of CNPase activity (fig.3, upper profile) corresponded to M_r 50 000 ± 8000 (low molecular weight enzyme form). This result is in good agreement with the estimations for CNPase polypeptides (51 -54 000) in SDS-polyacrylamide gel electrophoresis (fig.1). However, in presence of BSA the activity peak of CNPase appeared near the bottom of the gradient tube corresponding to a higher M_r of approx. 100 000 ± 8000 (fig.3, lower profile), which agrees well with the high molecular weight form obtained by gel filtration (fig.2). Minor shoulders of activity appearing in the lower mol. wt range of both gradients may represent partial degradation of the enzyme protein.

The high mol. wt enzyme form of approx. 100 000 (fig.3b) not only occurs in sucrose gradient analysis containing BSA (M_r 68 000), but also appears when the same analysis is carried out in the presence of other proteins of varying M_r values. e.g. human gamma globulin (150 000), carbonic anhydrase (29 000), or myelin basic protein (18 000) (unpublished). It is unlikely that the high mol. wt enzyme form represents an aggregate between a CNPase polypeptide and any of the added proteins, since the apparent molecular weights of these corresponding complexes would not coincide with M_r of 100 000. It is also noteworthy that the heavy enzyme form found during gel filtration was obtained in the absence of additional protein.

The persistence of the high mol. wt form of CNPase during gel filtration as well as in sedimentation rate analysis in the presence of BSA or other proteins makes it likely that CNPase has an M_r of about 100 000. This heavy enzyme form could be interpreted as being composed of two polypeptide chains with the apparent M_r values of 51 000 and 54 000 as separated by SDS-gel electrophoresis (fig.1) representing an M_r of approx. 105 000. The appearance of an M_r 50 000 peak of activity in sucrose density gradient analysis without BSA may indicate that the active site of the enzyme is associated with one (or both) of the dissociated polypeptide bands. The aggregate of two such subunits may be disrupted during sucrose density gradient centrifugation unless protected by additional protein. Possibly due to a deviation from the spherical shape, the mol. wt of the heavy enzyme form may be somewhat overestimated during gel filtration (M_r 120 000)

when compared with the sum of the molecular weight of the two polypeptides found in SDS gels. Assuming an M_r of about 100 000 for brain CNPase the molecular activity (turnover numbers) of our enzyme preparation for the hydrolysis of 2′,3′-cyclic nucleotides can be calculated from the appropriate specific activities given above. The apparent turnover numbers for cNADP, cAMP, cGMP, cCMP and cUMP are $25.0 \cdot 10^4$, $14.5 \cdot 10^4$, $9.0 \cdot 10^4$, $3.2 \cdot 10^4$ and $1.6 \cdot 10^4$, respectively.

For several reasons a comparison of some biochemical properties between the two types of enzymes CNPase and RNAase is of particular interest:

(a) CNPase fractionates very closely with RNAase activities in preparations from bovine spleen and liver [22] and the RNAase preparation from liver might be associated with a CNPase inhibitor [23].

(b) Both enzyme activities hydrolyze 2′,3′-cyclic nucleotide phosphodiesters, the known intermediates of RNA degradation [1,22]. However, unlike CNPase, the RNAase activities liberate the 3′-phosphomonoester from these cyclic nucleotides [1]. The K_m values estimated for pancreatic RNAase A are $6.9 \cdot 10^{-3}$ M (cUMP) and $3.5 \cdot 10^{-3}$ M (cCMP). For brain RNAase neither K_m values nor turnover numbers for the hydrolysis of 2′,3′-cyclic nucleotides have so far been reported. However, there is evidence for the existence of RNAases in brain, which hydrolyze RNA only to 2′,3′-cyclic nucleotides [25].

(c) Two catalytically active enzyme forms (monomeric and dimeric structures) have earlier been reported also for RNAases from different sources [26–28].

The physiological role of brain CNPase has not yet been elucidated, but there is some evidence for the possibility that this enzyme might be involved in the degradative or salvage pathway of RNA metabolism. It has been calculated [29] that CNS has a rapid turnover of RNA compared e.g. with liver. On the other hand, the functional role of the remarkably high CNPase activity in myelin fractions from CNS is still an open question.

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