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Chemical, immunological and catalytic properties of 2′:3′-cyclic nucleotide 3′-phosphodiesterase purified from brain white matter

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The amino acid composition, isoelectric point, specificity of the antibody raised and various catalytic properties were determined for 2′:3′-cyclic nucleotide 3′-phosphodiesterase (EC 3.1.4.37) purified from bovine brain white matter by a procedure involving solubilization with elastase (EC 3.4.21.11).

2′:3′-Cyclic nucleotide 3′-phosphodiesterase (EC 3.1.4.37) is a membrane-bound enzyme localized primarily in white matter of brain and spinal cord (Kurihara & Tsukada, 1967). The enzyme is firmly bound to the membrane structures of the white matter, and until recently the enzyme has resisted purification. We have purified the enzyme to homogeneity from bovine brain white matter by a procedure that involves solubilization by digestion with elastase (EC 3.4.21.11) (Nishizawa *et al.*, 1980). The purified enzyme was a monomer protein of mol.wt. approx. 30000. The present paper describes the chemical, immunological and catalytic properties of the purified enzyme.

Materials and methods

2′:3′-Cyclic AMP was prepared as described previously (Nishizawa *et al.*, 1980): 2′:3′-cyclic GMP, 2′:3′-cyclic CMP, 2′:3′-cyclic UMP, bovine serum albumin (fraction V) and *Escherichia coli* alkaline phosphatase (EC 3.1.3.1; chromatographed) were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Horse myoglobin, sperm-whale myoglobin, horse cytochrome *c* and a mixture of pI markers containing horse cytochrome *c* and its acetylated derivatives were purchased from Oriental Yeast Co., Tokyo, Japan. Ampholines were obtained from LKB-Produkter, Bromma, Sweden.

2′:3′-Cyclic nucleotide 3′-phosphodiesterase was purified from bovine brain white matter as described previously (Nishizawa *et al.*, 1980). The eluate from the affinity column was passed through a Sephadex G-25 column in 20mM-imidazole/HCl buffer (pH 6.7). The enzyme protein was determined as described previously (Nishizawa *et al.*, 1980) or by the amino acid analyses described below. The antiserum against 2′:3′-cyclic nucleotide 3′-phosphodiesterase was prepared as follows. The purified enzyme (0·2mg/ml) was emulsified with an equal volume of a 1:4 (v/v) mixture of complete and incomplete Freund's adjuvants. The emulsion was injected intracutaneously into New Zealand white rabbits; about 0.25mg of the purified enzyme was injected to each rabbit. Twice booster injections were given at 2-week intervals with incomplete Freund's adjuvant. The rabbits were bled about 1 month after the last injection.

For determination of the amino acid composition, the purified enzyme (85 μg) was hydrolysed in 6M-HCl (twice-distilled) at 110°C for 24h and 72h in an evacuated sealed ampoule. Amino acids were analysed with a JEOL amino acid analyser (JLC 6AS). For valine and isoleucine the values from 72h hydrolysis were used. For threonine and serine the values were extrapolated to zero time of hydrolysis. For the other amino acids (except cysteine, methionine and tryptophan) the average values from 24h and 72h hydrolyses were used. Cysteine and methionine were determined as cysteic acid and methionine sulphone respectively; the purified enzyme (85 μg) was oxidized with performic acid (Hirs, 1967) before hydrolysis. Tryptophan was determined after the purified enzyme (110 μg) was hydrolysed in 6M-HCl in the presence of 4% (v/v) thioglycollic acid (Matsubara & Sasaki, 1969).

Isoelectric focusing in polyacrylamide gel was carried out essentially as described by Miyazaki *et al.* (1976). Experimental conditions are given in the legend to Fig. 1.

The catalytic properties of 2′:3′-cyclic nucleotide 3′-phosphodiesterase were studied either by the

Abbreviation used: SDS, sodium dodecyl sulphate.

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potentiometric assay of Kurihara & Takahashi (1973) used at 30°C or by the colorimetric assay described in the legend to Fig. 3.

Results

Table 1 shows the amino acid composition of the purified enzyme. High lysine and leucine contents and a low methionine content were noted. The enzyme molecule contained 270 amino acid residues, of which 29 were lysine and 30 were leucine. The enzyme molecule contained only a single methionine residue. No amino sugar could be detected in the hydrolysate of the purified enzyme; therefore the purified enzyme may not be a glycoprotein. The amount of the purified enzyme determined by the amino acid analyses was approx. 70% of that determined by the colorimetric method of Lowry *et al.* (1951), where bovine serum albumin was used as a standard.

Isoelectric focusing of the purified enzyme was carried out at pH 7–11 in polyacrylamide gel. The purified enzyme migrated as a sharp protein band in the gel (see the insert to Fig. 1) and the final position of the protein band corresponded to pI 10.3–10.4 (Fig. 1).

Injection of the purified enzyme into rabbits effectively raised an antibody against the enzyme. When examined by the Ouchterlony double-immunodiffusion method, the serum yielded a single precipitin line with a crude enzyme preparation from bovine brain (Fig. 2, well A) or with the purified enzyme (Fig. 2, well B). The precipitin line formed with the crude enzyme and that formed with the purified enzyme were completely fused with each other. The serum gave a 'spur' between the purified enzyme (Fig. 2, well B) and a crude enzyme preparation from rat brain (Fig. 2, well C). The results indicate that the purified bovine enzyme and the crude bovine enzyme have identical antigenic

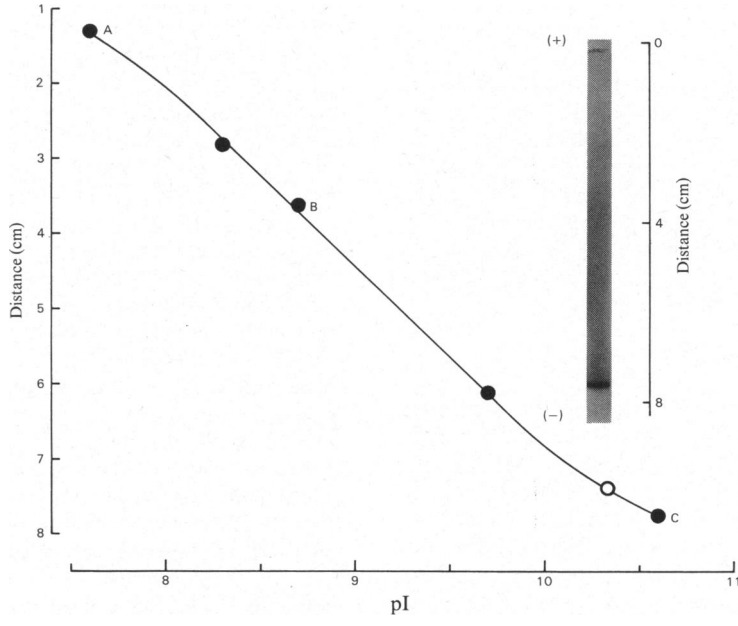

Fig. 1. *Isoelectric focusing of the purified 2′:3′-cyclic nucleotide 3′-phosphodiesterase*

A mixture containing 4.97% (w/v) acrylamide, 0.28% (w/v) *NN′*-methylenebisacrylamide, 2% (w/v) Ampholines (pH 7–9, 0.8%; pH 9–11, 0.8%; pH 3.5–10, 0.4%) and riboflavin (5 μg/ml) was photopolymerized in the presence of ammonium persulphate (0.2 mg/ml) and *NNN′N′*-tetramethylethylenediamine (0.6 μl/ml). Gel columns (8 cm long) were prepared in 0.5 cm-bore glass tubes. A sample solution (110–140 μl) containing 2% (w/v) Ampholines and 30% (w/v) sucrose was placed on top of the gel in the tube. Then 60 μl of 2% (w/v) Ampholines in 15% (w/v) sucrose was layered on top of the sample solution. The tube was finally filled with 0.01 M-H_3PO_4; 0.01 M-H_3PO_4 and 1 M-NaOH were used as anode and cathode solutions respectively. Electric current was applied for 5 h at 2°C with a constant voltage of 200 V. ○, 2′:3′-Cyclic nucleotide 3′-phosphodiesterase; ●, pI markers: A, horse myoglobin (pI 7.6); B, sperm-whale myoglobin (pI 8.7); C, horse cytochrome *c* (pI 10.6). A mixture of pI markers containing horse cytochrome *c* and its acetylated derivatives with pI values of 4.1, 4.9, 6.4, 8.3 and 9.7 was also used. Each point represents the mean of three to nine determinations. The pI value for the purified 2′:3′-cyclic nucleotide 3′-phosphodiesterase was 10.33 ± 0.06 (mean ± S.D. for eight determinations).

Table 1. *Amino acid composition of the purified 2′:3′-cyclic nucleotide 3′-phosphodiesterase*
Experimental details are given in the text.

Amino acid	Mole fraction (%)	Number of residues per molecule*
Asx	6.48	17.5
Thr	6.05	16.3
Ser	5.63	15.2
Glx	12.27	33.1
Pro	4.45	12.0
Gly	9.42	25.4
Ala	7.87	21.3
Cys	1.93	5.2
Val	5.79	15.6
Met	0.29	0.8
Ile	2.59	7.0
Leu	11.26	30.4
Tyr	2.93	7.9
Phe	4.12	11.1
Trp	2.15	5.8
Lys	10.71	28.9
His	1.68	4.5
Arg	4.36	11.8
Total	99.98	269.8

* A mol.wt. of 30000 (Nishizawa *et al.*, 1980) was used.

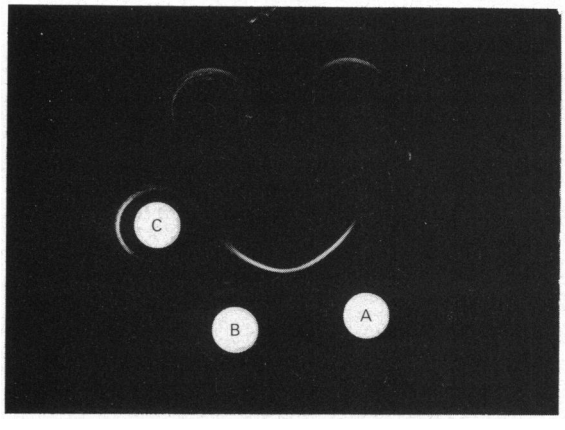

Fig. 2. *Ouchterlony double immunodiffusion of the rabbit antiserum prepared against the purified bovine 2′:3′-cyclic nucleotide 3′-phosphodiesterase*
The centre well was filled with the antiserum of 1:16 dilution (10 μl). Well A, crude bovine enzyme (2.2 units); well B, purified bovine enzyme (1.2 μg: 2.4 units); well C, crude rat enzyme (0.9 unit). For enzyme unit see Nishizawa *et al.* (1980). An Ouchterlony plate containing 1% (w/v) agar in 50mM-Tris/HCl buffer (pH 7.6) was used. Delipidated bovine brain white matter (Kurihara *et al.*, 1977) was digested with elastase as described previously (Nishizawa *et al.*, 1980) and the supernatant fluid was used as the crude bovine enzyme. Delipidated rat whole brain was prepared and treated similarly and the supernatant fluid was used as the crude rat enzyme.

determinants, at least for the antibody used, and that the bovine and rat enzymes have partially common antigenic determinants.

The enzyme reaction of the purified 2′:3′-cyclic nucleotide 3′-phosphodiesterase proceeded linearly with time in the presence of serum albumin, hexadecyltrimethylammonium bromide or Triton X-100. However, if these reagents had not been added to the reaction mixture, the reaction velocity rapidly fell with time.

The K_m values for the purified enzyme were determined by a potentiometric assay (Kurihara & Takahashi, 1973), the most reliable assay for K_m determination. The K_m values with 2′:3′-cyclic AMP, 2′:3′-cyclic GMP, 2′:3′-cyclic CMP and 2′:3′-cyclic UMP in the presence of 0.2% serum albumin (or 0.2% hexadecyltrimethylammonium bromide) were 0.10 (0.22)mM, 0.40 (0.57)mM, 6.3 (5.6)mM and 7.1 (8.3)mM respectively. The K_m value with 2′:3′-cyclic AMP in the presence of hexadecyltrimethylammonium bromide (0.22mM) was the same as that determined previously for a suspension of white matter in this detergent (0.25mM; Nishizawa *et al.*, 1980).

The purified enzyme had an optimum pH at pH 6.0 in the presence of both hexadecyltrimethylammonium bromide and serum albumin (Fig. 3*a*). If the detergent was omitted, the pH–activity profile was less sharp and an optimum pH ranging from 5.4 to 6.2 was observed (Fig. 3*a*). The pH–activity profile for the enzyme in delipidated white matter determined in the presence of both hexadecyltrimethylammonium bromide and serum albumin was almost the same as that for the purified enzyme. The progress curves at 20°C, 30°C, 37°C and 45°C (Fig. 3*b*) showed that the reaction velocity was maximum at 37°C. At 45°C the velocity declined with time, suggesting that inactivation occurred during the reaction. The enzyme reaction was highly ionic-strength-dependent if hexadecyltrimethylammonium bromide was omitted from the reaction mixture (Fig. 3*c*). The activity of the purified enzyme at 30°C under the optimum conditions (10mM-imidazole/HCl buffer at pH 6.0, 0.2% hexadecyltrimethylammonium bromide) was approx. 4.0mmol/min per mg of enzyme protein determined by the amino acid analyses or 120mmol/min per μmol of enzyme protein.

Discussion

Guanidinium chloride (Guha & Moore, 1975; Drummond *et al.*, 1978; Drummond, 1979; Sprinkle *et al.*, 1980), Triton X-100/salt (Suda & Tsukada, 1980) and elastase (Nishizawa *et al.*, 1980) have been used to solubilize 2′:3′-cyclic nucleotide 3′-phosphodiesterase. Drummond *et al.* (1978) first obtained a nearly homogeneous enzyme preparation

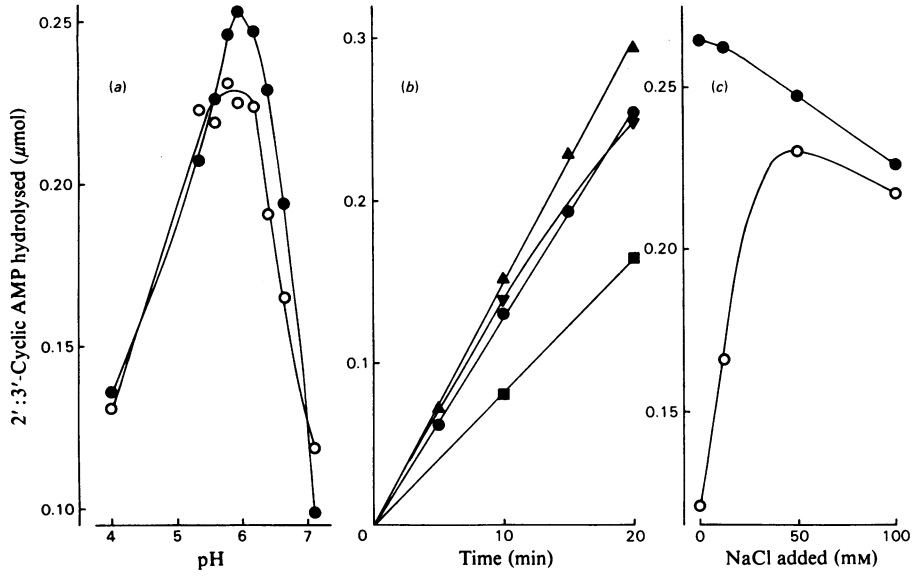

Fig. 3. *Effects of pH (a), temperature (b) and ionic strength (c) on the enzyme reaction*

The purified 2':3'-cyclic nucleotide 3'-phosphodiesterase (239 units/ml; for enzyme unit see Nishizawa *et al.*, 1980) in 0.1% serum albumin was diluted 1000-fold with 0.1% serum albumin and 50 μl of the diluted enzyme solution was used for each activity determination. The reaction mixture (200 μl) in a plastic tube contained both 0.2% hexadecyltrimethylammonium bromide and 0.05% serum albumin (■, ●, ▲, ▼), or 0.05% serum albumin alone (○), in addition to 50 mM [10 mM for (*c*)] imidazole/HCl buffer (pH 6.0), 7.5 mM-2':3'-cyclic AMP and the enzyme solution. The reaction mixture for (*c*) also contained various concentrations of NaCl. The reaction mixture was incubated at 20°C (■), 30°C (●, ○), 37°C (▲) or 45°C (▼); incubation time was 20 min in (*a*) and (*c*). The reaction was stopped by addition of 100 μl of 0.2 M-Na_2CO_3. A 50 μl portion of the mixture was taken and incubated for 1 h at 37°C with 100 μl (0.5 unit) of *Escherichia coli* alkaline phosphatase. Then 1 ml of a freshly prepared 'mixed reagent' containing 0.5% (w/v) $(NH_4)_6Mo_7O_{24}4H_2O$, 0.9 M-H_2SO_4, 2% (w/v) L-ascorbic acid and 2% (w/v) SDS was added: the total volume was adjusted to 2 ml with water. Colour was developed by heating for 40 min at 45°C and the A_{830} was measured. To the reaction mixture without the enzyme solution (150 μl), 100 μl of 0.2 M-Na_2CO_3 and then 50 μl of the enzyme solution was added. A 50 μl portion of the mixture was treated as above and the A_{830} obtained was used as a blank. The A_{830} for 0.05 μmol of KH_2PO_4 was used as a P_i standard. Each point represents the mean of duplicate or triplicate determinations.

from bovine brain white matter by the guanidinium chloride method. The major protein in their preparation had a mol.wt. of 100000 by gel filtration and 50000 by SDS/polyacrylamide-gel electrophoresis, and appeared to be a dimer protein composed of two identical subunits. More recent studies, however, have shown that two protein bands of only slightly different mobilities can be discerned by SDS/polyacrylamide electrophoresis of higher resolving power, e.g. by the procedure of Laemmli (1970) (Drummond, 1979; Sprinkle *et al.*, 1980). The enzyme protein prepared in our laboratory from bovine brain white matter by the elastase method had a mol.wt. of 31000 by gel filtration and 29000 by SDS/polyacrylamide-gel electrophoresis (Nishizawa *et al.*, 1980). This protein migrates as a single protein band on SDS/polyacrylamide-gel electrophoresis after the procedure of Laemmli (1970).

The purified enzyme protein prepared by the elastase method had an amino acid composition very similar to that of the preparation obtained by Drummond *et al.* (1978). Methionine and cysteine contents, however, seem to be significantly lower in our preparation. It was particularly noted that the enzyme molecule contains only a single methionine residue. The purified enzyme was highly basic, giving a sharp protein band at pH 10.3–10.4 on isoelectric focusing. In contrast, the enzyme proteins prepared by the guanidinium chloride and Triton X-100/salt methods distributed diffusely around pH 9 on isoelectric focusing (Sprinkle *et al.*, 1980; Suda & Tsukada, 1980).

The purified enzyme described here may be a fragment of the native enzyme produced by digestion with elastase. However, this preparation effectively raised an antibody in rabbits. As far as we have examined in the present study, the catalytic

properties of the enzyme remain unchanged during the purification. Although the purified enzyme is inactivated by dilution or freezing-and-thawing (Nishizawa *et al.*, 1980) and requires the presence of serum albumin or detergents to give a linear progress curve of the enzyme reaction, these phenomena may be due to a decrease in enzyme stability by isolation and are sometimes encountered for other enzymes.

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