Role of Neuraminic Acid in the Heterogeneity of Alkaline Phosphatase in Sheep Brain

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1. Two alkaline phosphatase fractions from sheep brain obtained by DEAE-cellulose column chromatography were shown to be associated with different concentrations of NANA (*N*-acetylneuraminic acid). Enzyme II contains nearly three times as much NANA as does enzyme I. 2. Partial removal of NANA by neuraminidase digestion from these alkaline phosphatase fractions has different effects on their chromatographic properties. Though the enzymic release of NANA has no effect on the elution pattern of enzyme I from a DEAE-cellulose column, such a treatment shifts the elution pattern of enzyme II towards that of enzyme I. 3. However, this change in the elution pattern of enzyme II as a result of the removal of NANA does not produce any change in the kinetics of this fraction, and the differences between enzyme I and enzyme II with respect to their substrate affinities and K_i for phosphate inhibition are maintained even after the removal of NANA. 4. Results indicate that NANA is not the only factor responsible for the heterogeneity of alkaline phosphatase in sheep brain and enzyme I is not the result of the removal of NANA from enzyme II.

Recent observations by many investigators that neuraminidase treatment results in a decreased anodal migration of some of the alkaline phosphatase fractions during electrophoresis have led to the suggestion that the fundamental difference between the alkaline phosphatase isoenzymes may lie in the different amounts of NANA* associated with them (Robinson & Pierce, 1964; Butterworth & Moss, 1966; Moss, Eaton, Smith & Whitby, 1966).

Earlier work from our Laboratory has indicated the presence in sheep brain of two alkaline phosphatase fractions that can be separated by chromatography on DEAE-cellulose and that show small but definite differences in some of their properties (Saraswathi & Bachhawat, 1966).

In view of the recent observations implicating NANA as one of the factors responsible for the heterogeneity of alkaline phosphatase, a study was undertaken to assess the role of NANA in the heterogeneity of alkaline phosphatase in sheep brain. In the present paper the effect of the enzymic removal of NANA on the chromatographic pattern as well as kinetics of the alkaline phosphatase fractions from sheep brain is described. The results indicate that, even though NANA may be responsible for the higher negative charge of one of the alkaline phosphatase fractions, this is not

the only factor responsible for the heterogeneity of alkaline phosphatase in sheep brain.

MATERIALS AND METHODS

Materials. Pyridoxal phosphate, *p*-nitrophenyl phosphate, AMP and *Vibrio comma* (formerly *Vibrio cholerae*) neuraminidase (crude cholera filtrate) were purchased from Sigma Chemical Co., St Louis, Mo., U.S.A. Sephadex G-200 was purchased from Pharmacia, Uppsala, Sweden, and DEAE-cellulose from Carl Schleicher and Schuell Co., Keene, N.H., U.S.A.

Preparation of isoenzymes. A 200 g. portion of fresh sheep brain cortex was homogenized with 500 ml. of 0.15 M-NaCl for 30 sec. in a Waring Blender. The homogenate was centrifuged at 1000g for 10 min. and the precipitate, which contained about 85% of alkaline phosphatase activity of original homogenate, was rehomogenized with 400 ml. of 0.1 M-sodium acetate buffer, pH 5.0.

The extraction of alkaline phosphatase from the above homogenate by Morton's (1954) butanol method, as well as the separation of the two isoenzymes (enzyme I and enzyme II) by chromatography on DEAE-cellulose, was done as reported previously (Saraswathi & Bachhawat, 1966). Since only 10 mg. of protein was employed, micro-columns (0.5 cm. $\times$ 10 cm.) of DEAE-cellulose were used. The same gradient elution was performed with increasing concentration of chloride ion in tris-HCl buffer, pH 7.4. Effluent was collected in 0.5 ml. portions, a rate of flow of 1 ml./4 min. being maintained.

A 25 mg. sample of enzyme I and a 36 mg. sample of

* Abbreviation: NANA, *N*-acetylneuraminic acid.

enzyme II were passed through a column (2.2 cm. $\times$ 50 cm.) of Sephadex G-200. Enzyme I and enzyme II were eluted from the column by 0.05 M-tris-HCl buffer, pH 7.4 (see Fig. 1), and concentrated by freeze-drying. Enzymes at this stage were designated as enzyme I (G-200) and enzyme II (G-200) respectively.

Digestion with neuraminidase. A 10 mg. sample of enzyme I (G-200) and a 12 mg. sample of enzyme II (G-200) were incubated for 6 hr. with 5 mg. of neuraminidase dissolved in 0.5 ml. of 0.05 M-sodium acetate buffer, pH 5.5, containing NaCl (0.06 M) and CaCl_2 (4 mM) in a total volume of 1 ml. Neuraminidase was omitted from the control tubes.

The incubation mixtures were passed through columns of Sephadex G-200. Elution of alkaline phosphatase and concentration were carried out as described above. After dialysis for 6 hr. against 0.05 M-tris-HCl buffer, pH 7.4, the four incubation mixtures were rechromatographed on a DEAE-cellulose column with gradient elution as described by Saraswathi & Bachhawat (1966). The major alkaline phosphatase fractions from neuraminidase-treated enzyme I and enzyme II were designated as enzyme IA and enzyme IIA respectively, and the corresponding peaks from untreated controls were designated as enzyme IB and enzyme IIB respectively.

Assay for neuraminic acid. Concentration of protein-bound NANA was estimated after hydrolysis in the presence of 0.1 N- H_2SO_4 for 1 hr. at 80° by determining the neuraminic acid released by the thiobarbituric acid method of Warren (1959) as modified by Saifer & Gerstenfeld (1962).

Assay of enzyme activity. Alkaline phosphatase activity was assayed with *p*-nitrophenyl phosphate as substrate. The incubation mixture contained 0.5 μ mole of the substrate, 50 μ moles of tris-HCl buffer, pH 9.0, 0.5 μ mole of MgCl_2 and the enzyme in a total volume of 0.5 ml. for 30 min.; the colour due to *p*-nitrophenol was developed by adding 2.5 ml. of 2 N-NaOH and readings were taken in a Klett colorimeter with a 42 filter. Whenever pyridoxal phosphate was used as substrate, the method of Wada & Snell (1961) was employed, as described previously (Saraswathi & Bachhawat, 1963).

Inorganic phosphate was assayed by the method of Fiske & Subbarow (1925). Protein concentrations were determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

One unit of enzyme was defined as 1 μ mole of *p*-nitrophenyl phosphate cleaved/min. Specific activity was expressed as units/mg. of protein.

RESULTS

Effects of neuraminidase treatment on elution patterns. Table I shows the specific activity as well as the NANA content of enzyme I and enzyme II during various steps. Enzyme II has nearly three times as much NANA associated with it as has enzyme I. Incubation with neuraminidase releases 65% of NANA in each case. The activities of both the enzymes remain unaffected by the release of NANA. Gel filtration on Sephadex G-200 produces a twofold increase in specific activity as well as in NANA/mg. of protein in each case.

Fig. 1 shows the elution patterns of enzyme I and enzyme II from Sephadex G-200 after incubation with and without neuraminidase. Whereas enzyme I and enzyme II have different elution patterns from the Sephadex G-200 column, the removal of NANA does not produce any change in their respective elution patterns. This indicates that during neuraminidase digestion there was no significant change in the molecular weights of the alkaline phosphatase fractions. Moreover, gel filtration on Sephadex G-200 facilitates the removal of neuraminidase from the incubation mixtures, as the molecular weight of *Vibrio comma* neuraminidase has been shown to be 20000 (Ada & French, 1959; Schramm & Mohr, 1959). This was confirmed

Table 1. *Specific activities and NANA contents of enzyme I and enzyme II at various steps during purification*

Incubation with neuraminidase was carried out as described in the text. Enzyme IA, enzyme IIA, enzyme IB and enzyme IIB are defined in the text.

Enzyme	Sp. activity (units/mg. of protein)	NANA content (μ moles/mg. of protein)
Enzyme I	0.033	3.6
Enzyme I (G-200)	0.060	6.0
Enzyme I (G-200) + neuraminidase	0.060	1.0
Enzyme I (G-200) - neuraminidase	0.060	5.0
Enzyme IA	0.067	—
Enzyme IB	0.067	—
Enzyme II	0.067	9.0
Enzyme II (G-200)	0.17	21.0
Enzyme II (G-200) + neuraminidase	0.16	8.5
Enzyme II (G-200) - neuraminidase	0.16	17.0
Enzyme IIA	0.18	0.0
Enzyme IIB	0.18	12.0

Fig. 1. Elution patterns from Sephadex G-200 of enzyme I (a) and enzyme II (b) with and without neuraminidase treatment. Gel filtration on Sephadex G-200 was carried out as described in the text; the various fractions were assayed with *p*-nitrophenyl phosphate as substrate. Δ , Enzyme I—neuraminidase; $\blacktriangle$, enzyme I+neuraminidase; $\circ$, enzyme II—neuraminidase; $\bullet$, enzyme II+neuraminidase.

Fig. 2. Elution patterns of enzyme I (a) and enzyme II (b) from a DEAE-cellulose micro-column (0.5 cm. $\times$ 10 cm.) with and without the removal of NANA. Chromatography on DEAE-cellulose and gradient elution were carried out as described by Saraswathi & Bachhawat (1966). Δ , Enzyme I or enzyme II after neuraminidase treatment; $\bullet$, enzyme I or enzyme II without neuraminidase treatment.

by testing these fractions for neuraminidase activity with enzyme II as substrate as described above.

Fig. 2 shows the effect of the removal of NANA on the elution patterns of enzyme I and enzyme II from a DEAE-cellulose column. Whereas the removal of NANA does not produce any change in the elution pattern of enzyme I, a major portion of enzyme II is eluted at a much lower salt concentration after the removal of NANA, and the chromatographic pattern becomes almost identical

with that of enzyme I. Moreover, it was also observed that after the removal of NANA the mobility of enzyme II during paper electrophoresis, as described previously (Saraswathi & Bachhawat, 1966), becomes almost identical with that of enzyme I.

Effects of neuraminidase treatment on kinetics. The observation that the removal of NANA shifts the elution pattern of enzyme II towards that of enzyme I led us to investigate the kinetics of these alkaline phosphatase fractions after the removal of

Fig. 3. Lineweaver-Burk plots of enzyme IA (a) and enzyme IB (b) in the presence and absence of phosphate. The incubation mixture consisted of 50 μ moles of tris-HCl buffer, pH 9.0, 5 μ moles of $MgCl_2$, the indicated concentration of pyridoxal phosphate, the enzyme fraction as indicated and where mentioned 2 mM-phosphate in a total volume of 0.5 ml. After incubation for 30 min. the disappearance of pyridoxal phosphate was measured as described in the text. ●, Enzyme IA—phosphate; ○, enzyme IA+phosphate; ◆, enzyme IB—phosphate; ◇, enzyme IB + phosphate.

Fig. 4. Lineweaver-Burk plots of enzyme IIA (a) and enzyme IIB (b) for pyridoxal phosphate in the presence and absence of phosphate. ●, Enzyme IIA—phosphate; ○, enzyme IIA+phosphate; ◆, enzyme IIB—phosphate; ◇, enzyme IIB+phosphate.

Table 2. *Kinetics of pyridoxal phosphate cleavage by neuraminidase-treated enzyme I and enzyme II and by the untreated controls*

Enzyme	$10^4 \times K_m$ (pyridoxal phosphate) (M)	$10^4 \times \text{Concn. of}$ pyridoxal phosphate at $V_{\max}$ (M)	$10^4 \times K_i$ (M)
Enzyme IA	1.8	3.0	8.5
Enzyme IB	2.0	5.0	8.0
Enzyme IIA	4.5	10.0	42.0
Enzyme IIB	5.0	12.0	50.0

Fig. 5. K_m values for AMP of enzymes IA and IB (a) and of enzymes IIA and IIB (b). The incubation mixture consisted of 50 μ moles of tris-HCl buffer, pH 9.0, 5 μ moles of $MgCl_2$, AMP of the indicated concentration and different enzyme fractions as indicated in a total volume of 0.5 ml. After incubation for 30 min. the inorganic phosphate liberated was determined as described in the text. $\diamond$, Enzyme IA; $\blacklozenge$, enzyme IB; $\circ$, enzyme IIA; $\bullet$, enzyme IIB.

NANA, to see whether the presence of NANA is responsible for the existence of two alkaline phosphatases in sheep brain. We have shown previously (Saraswathi & Bachhawat, 1966) that enzyme I and enzyme II differ mainly in their affinity towards pyridoxal phosphate and AMP as well as in their K_i for phosphate inhibition. Hence these properties were investigated on the major alkaline phosphatase fractions obtained by the DEAE-cellulose column chromatography of neuraminidase-treated enzyme I and enzyme II as well as the controls.

Figs. 3 and 4 show the Lineweaver-Burk plots of these alkaline phosphatase fractions for pyridoxal phosphate. Curves obtained in presence of 2 mM-phosphate are also shown. The K_m value for pyridoxal phosphate, the pyridoxal phosphate concentration at $V_{\max}$, as well as K_i for phosphate inhibition, calculated from the data of Figs 3 and 4, are shown in Table 2. It is evident that the

removal of NANA does not produce any change in the above properties of enzyme I and enzyme II, and the differences between these two alkaline phosphatase fractions with respect to K_m and $V_{\max}$ for pyridoxal phosphate as well as K_i are maintained even after the removal of NANA from these enzyme fractions.

Fig. 5 shows that enzyme II has a lower affinity for AMP compared with enzyme I irrespective of whether NANA was removed from these enzymes or not.

DISCUSSION

Although earlier investigators have reported that neuraminidase treatment decreases the electrophoretic mobilities of certain alkaline phosphatases, there has been no direct evidence to indicate the association of NANA in these enzymes. The present results indicate that, even though two alkaline phosphatase fractions from sheep brain

differ in the amount of NANA associated with them, this is by no means the only factor responsible for the heterogeneity of alkaline phosphatase in brain. The higher amount of NANA associated with enzyme II may contribute to its negative charge, as the removal of NANA results in the elution of this enzyme at a lower salt concentration.

NANA apparently does not constitute a part of the active site of enzyme I and enzyme II, as the removal of NANA does not affect the activities of these enzymes. Similar observations have been made for kidney alkaline phosphatase fractions by Butterworth & Moss (1966) and for liver alkaline phosphatase by Moss *et al.* (1966), who reported that incubation with neuraminidase decreases the electrophoretic mobilities of these enzymes without affecting their activity.

The observation that neuraminidase treatment essentially eliminates the differences in the electrophoretic mobilities of various alkaline phosphatase fractions from kidney has led to the suggestion (Butterworth & Moss, 1966) that the different alkaline phosphatase fractions obtained on electrophoresis arise as a result of the loss of NANA from one fraction during the extraction procedure. The present results, however, indicate that the two alkaline phosphatase fractions from sheep brain are two different enzymes and that enzyme I is not the result of the splitting of NANA from enzyme II, as the small differences in the properties of enzyme I and enzyme II are maintained even after the removal of NANA from these enzymes.

The presence of neuraminidase in several mammalian tissues has been reported by many investigators (Warren & Spearing, 1962; Ada & Lind, 1961; Carubelli, Trucco & Caputto, 1962; Taha & Carubelli, 1967). Of particular interest is the report of Morgan & Laurell (1963) that homogenates prepared from human, guinea-pig and ox brains release sialic acid at 37°, and this has been attributed

to the presence of neuraminidase in brain. In view of these reports indicating the presence of neuraminidase activity in brain, the possibility exists that enzyme I may contain small amounts of enzyme II from which NANA has been split off by the neuraminidase present in brain.

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