Short Communications

Enzymic Degradation of Cytidine 5'-Monophosphate N-Acetylneuraminic Acid

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The role of CMP-NANA* as a donor of the NANA moiety of ganglioside (Kanfer, Blacklow, Warren & Brady, 1964), colominic acid (Aminoff, Dodyk & Roseman, 1963), neuramin-lactose (Jourdan, Carlson & Roseman, 1963) and glycoproteins (Roseman, 1964; O'Brien, Canady, Hall & Neufeld, 1966) is well recognized. The formation of *N*-acetylmannosamine from UDP-*N*-acetylglucosamine by UDP-*N*-acetylglucosamine 2-epimerase is inhibited by CMP-NANA (Kornfeld, Kornfeld, Neufeld & O'Brien, 1964). Thus the concentration of CMP-NANA plays an important role in regulating various synthetic pathways. Attempts to isolate CMP-NANA from mammalian tissues have been unsuccessful; however, occasionally free CMP and NANA were found in nearly equimolar quantities in hog submaxillary-gland extract (Jourdan & Roseman, 1963). CMP-NANA is completely resistant to digestion by alkaline phosphatase (EC 3.1.3.1) of *Escherichia coli* and intestine, venom phosphodiesterase (EC 3.1.4.1), 5'-nucleotidase (EC 3.1.3.5) and also phosphodiesterase (EC 3.1.4.1) and the phosphatases (EC 3.1.3.1) together (Warren & Blacklow, 1962) as well as neuraminidase (EC 3.2.1.18) (Comb, Shimizu & Roseman, 1959). These reports led us to study the enzymic degradation of CMP-NANA.

In the present communication evidence is presented to demonstrate the presence of an enzyme that cleaves CMP-NANA. The enzyme has been partially purified from sheep liver and its properties have been studied.

Materials and methods. CMP-NANA was prepared enzymically by using sheep-brain enzyme purified up to the second ammonium sulphate stage (Shoyab, Pattabiraman & Bachhawat, 1964). CMP-NANA was isolated on a DEAE-cellulose column as described by Warren & Blacklow (1962). The fractions containing CMP-NANA were pooled together and concentrated under vacuum. Lithium chloride was removed by passing through a Bio-Gel P-2 column.

The degrading enzyme was assayed by measure-

ment of the disappearance of CMP-NANA. The reaction mixture for the estimation of the enzyme activity consisted of 0.07 μ mole of CMP-NANA, 0.02 μ mole of $MnSO_4$, 30 μ moles of tris-acetate buffer, pH 9.0, and the enzyme in a total volume of 0.2 ml. After 1 hr. incubation at 37°, the remaining CMP-NANA was determined in the presence of NANA as described by Shoyab *et al.* (1964). Two control experiments were carried out, one having all the components of the reaction mixture minus CMP-NANA and the other lacking enzyme to correct the contribution of colour by the enzyme itself, since, in the initial stages of purification, even after treatment with $NaBH_4$, the enzyme gave some colour with thiobarbituric acid.

Purification of the enzyme. The frozen sheep liver was homogenized with 5 vol. (w/v) of 0.05 M-tris-acetate buffer, pH 7.6, the pellet obtained after centrifugation at 5000g for 20 min. was suspended in the same buffer and this was extracted with 20% (v/v) butanol. The aqueous butanol layer was dialysed against 0.01 M-tris-acetate buffer, pH 7.6. The supernatant solution was brought to 60% saturation with ammonium sulphate, and the precipitate was dissolved in tris-acetate buffer, pH 7.6, and dialysed for 2 hr. against the same buffer. The pH of the dialysed solution was adjusted to 4.6 with *N*-acetic acid and left to stand for 1 hr. The precipitate was discarded and the supernatant liquid was adjusted to pH 7.6 with M-tris-acetate buffer, pH 7.6. The enzyme was precipitated by adjusting the ethanol and Zn^{2+} concentrations to 20% and 5 mM respectively. The precipitate was suspended in 0.02 M-tris-acetate buffer, pH 7.6. The suspension was subjected to ammonium sulphate fractionation and the precipitate obtained at 40–80% saturation was dissolved in the minimum volume of 0.02 M-tris-acetate buffer, pH 7.6. The solution was passed through a Sephadex G-200 column. The fractions containing enzyme activity were pooled together and subjected to DEAE-cellulose column chromatography. The enzyme was eluted from the column with 0.4 M-tris-acetate buffer, pH 7.6.

Results. By using the procedure described above

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

there was an overall recovery of 4.5% of the original activity and 173-fold purification was achieved. With the purified enzyme 1223 μ moles of CMP-NANA were cleaved/mg. of enzyme protein. The purified enzyme could be stored for 1 month either at -18° or 0° without any appreciable loss of activity. The enzyme preparation was contaminated with alkaline phosphatase (EC 3.1.3.1), 5'-nucleotidase (EC 3.1.3.5) and ATP diphosphohydrolase (EC 3.6.1.5).

The kinetic studies showed that: (a) the enzymic activity was linear with time and protein concentration; (b) the pH optimum in tris-acetate buffer was 9.0; (c) the approximate K_m value for CMP-NANA was 1×10^{-4} M; (d) the stoichiometry of the reaction showed that 1 mole each of NANA and P_i were formed with the concomitant disappearance of 1 mole of CMP-NANA; (e) the enzyme did not have an absolute requirement for a metal ion, though the activity was increased in the presence of Mn^{2+} and Mg^{2+} ; (f) the enzyme was completely inhibited by EDTA, the inhibition being reversed by Mn^{2+} but not by Mg^{2+} . The enzyme was markedly inhibited by thiols (cysteine, glutathione, β -mercaptoethanol and 2,3-dimercaptoethanol) in the absence of Mn^{2+} , but the inhibition was less prominent in the presence of Mn^{2+} . The enzyme was markedly inhibited by nucleotide mono-, di- and tri-phosphates, and complete inhibition was observed at 0.5 mM-ATP concentrations.

The products of the enzymic hydrolysis of CMP-NANA were identified as cytidine by paper chromatography: NANA by paper chromatography and thiobarbituric acid assay with and without $NaBH_4$ -acetone treatment, and P_i by the method of Fiske & Subbarow (1925). Studies on the subcellular distribution of the enzyme indicated that most of the activity was located in the nuclei and mitochondria. The enzyme was present in all the rat tissues investigated, i.e. kidney, liver, cartilage, intestine, lung, heart, spleen, brain and skeletal muscle. Under the assay condition liver, kidney and cartilage had higher specific activity than other tissues.

Discussion. Owing to the presence of CMP phosphohydrolase (EC 3.1.3.5) in the purified enzyme, it has not been possible to establish the sequence of reactions leading to the formation of

cytidine, NANA and P_i . On the basis of the observation reported, the enzymic cleavage of CMP-NANA may occur by one of the three mechanisms:

Attempts to inhibit CMP phosphohydrolase (EC 3.1.3.5) by the use of various reagents were unsuccessful because a substantial inhibition of CMP-NANA-degrading enzyme was also observed under these conditions.

The physiological role of this enzyme is not known at the present time. However, it is likely that the degrading enzyme has a regulatory function in the formation of NANA and NANA-containing compounds.

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