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PROPERTIES OF GLYCOSYL TRANSFER ENZYMES OF BOVINE RETINA

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SUMMARY

1. The enzyme catalyzing the transfer of *N*-acetylneuraminic acid (NANA) to an endogenous acceptor exhibits a K_m of 7 μM for CMP–NANA. The transfer of NANA is stimulated by CTP and glutathione and by UDP–galactose.

2. The galactose transfer enzyme exhibits a K_m of 6.5 μM for UDP–galactose. The transfer of galactose is stimulated by UTP and requires $MnCl_2$ (2.5–5 mM).

3. UDP–galactose-stimulated NANA transfer also requires $MnCl_2$ (2.5 mM).

INTRODUCTION

In a previous paper[1] we described the sequential addition of glycosyl residues to the carbohydrate chains of glycoprotein in a particulate fraction of bovine retina. Enzymes are present that catalyze the transfer of *N*-acetylneuraminic acid (NANA) from CMP–NANA and galactose from UDP–galactose to acceptors also bound to the particulate preparation. The present paper describes some of the properties of these transfer enzymes, the cofactor requirements and additional evidence for the sequential transfer of galactose and NANA.

EXPERIMENTAL

Materials

CMP–NANA, prepared as previously described[1] was labeled in the acetyl group with [^{3}H]acetic anhydride (Nuclear Chicago). UDP–[^{14}C]galactose was purchased from International Chemical and Nuclear. Other nucleotides and glutathione were purchased from Sigma, and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) from Calbiochem.

Abbreviations: NANA, *N*-acetylneuraminic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

Analytical methods

Protein was determined by the method of LOWRY *et al.*[2]. Radioactivity was measured with a liquid scintillation spectrometer using a toluene phosphor (0.4% 2,5-diphenyl-oxazole and 0.005%, 1,4-bis-2'[5-phenyloxazolyl]benzene in toluene).

Paper chromotography was carried out on Whatman No. 1 paper in the following solvents: I (isoamyl acetate–acetic acid–water, 3:3:1 by vol.), II (pyridine–ethyl acetate–water, 1:3.6:1.15 by vol.). Radioactivity on paper chromatograms was measured with a 4-π chromatogram scanner.

Enzymes

Eyes were removed from cattle immediately after slaughter and were stored on ice until dissected. All subsequent operations were carried out at 0–4°. Retinas were removed, washed and homogenized as previously described[1], using a Tenbroek homogenizer followed by a Potter–Elvehjem homogenizer. The final suspension contained 4 mg protein in 0.1 ml.

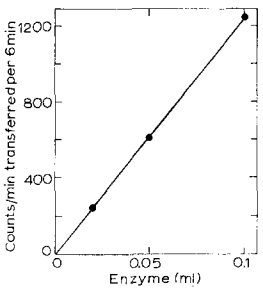
Pronase was purchased from Calbiochem and crystalline hog pancreatic α-amylase from Sigma.

Assay of NANA transfer

CMP-*N*-[^{3}H]acetylneuraminic acid (0.433 nmole, 65 000 counts/min) was incubated at 37° with 0.1 ml enzyme in a volume of 0.225 ml containing 2.5 mM $MnCl_2$, 5 mM glutathione, 0.3 mM CTP, 0.5 mM UTP and 50 mM HEPES buffer (pH 6.3). Variables or additions are indicated in the figures. Transfer of NANA from CMP–NANA to endogenous acceptor was measured as trichloroacetic acid-insoluble radioactivity. Reactions were stopped by the addition of 2 ml cold 5% trichloroacetic acid. Samples were prepared and counted as previously described[1]. All experimental values are corrected for zero time controls, usually about 10 counts/min above background.

Assay of galactose transfer

UDP–[^{14}C]galactose (0.84 nmole, 33 000 counts/min) was incubated and reaction mixtures were precipitated in the same way as described for CMP–[^{3}H]NANA. However, radioactivity incorporated into glycogen must be removed before trichloroacetic acid precipitation. At the times indicated in the figures the individual tubes were chilled in ice and the following additions were made to all tubes including the zero-time control: 52 nmoles unlabeled UDP–galactose, 0.1 ml 1.5% deoxycholate in 50 mM HEPES buffer (pH 6.3) and 350 units[3] α-amylase. All tubes were then reincubated for 3 h at 37°. The dilution of label by UDP–galactose and the disruption of the membranous particles by deoxycholate combine to prevent any further incorporation of galactose while the amylase destroys labeled glycogen. Zero-time controls treated and incubated in this way average 35 counts/min above background, only 15 counts/min higher than controls not further treated. Hydrolysis of the trichloroacetic acid-insoluble product at 100° in 1 M H_2SO_4 for 4 h released all the radioactivity which co-chromotographed on paper with carrier galactose in Solvents I and II. No trace of [^{14}C]glucose could be detected. Without amylase treatment, as much as 60% of the radioactivity coincided with carrier glucose.



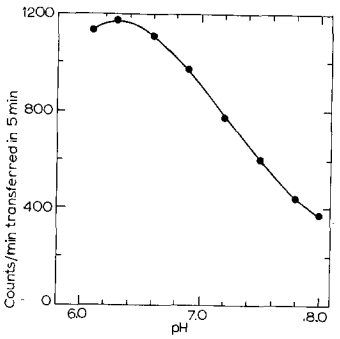


Fig. 1. Effect of enzyme concentration on the rate of NANA transfer. Incubation mixtures are as described under *Assay of NANA transfer* with enzyme as indicated. Protein concentration is 40 mg/ml.

Fig. 2. Effect of pH on the rate of NANA transfer. Incubation mixtures are as described under *Assay of NANA transfer* with HEPES buffer of the pH indicated.

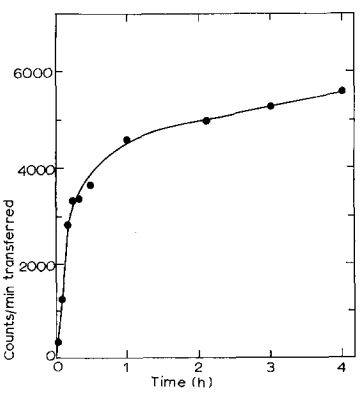
RESULTS

Properties of CMP–N-acetylneuraminic acid: glycoprotein sialyl transferase

Transfer of NANA is proportional to the concentration of the particulate fraction (Fig. 1) and has a pH optimum of 6.3 in HEPES buffer (Fig. 2) or in imidazole–HCl (not shown), with a somewhat lower rate in the latter.

NANA transfer follows a bimodal time curve with a rapid initial incorporation which is linear for 10 min and a slow incorporation, linear for at least 4 h (Fig. 3). All studies reported here are concerned with the initial rapid transfer of NANA.

The transfer reaction is stimulated by CTP (Fig. 4) with a maximum between 0.15 and 0.45 mM. Glutathione also stimulates (Fig. 5) but only when CTP is present. Stimulation by these cofactors would be expected if CMP sialic acid synthetase[4] were



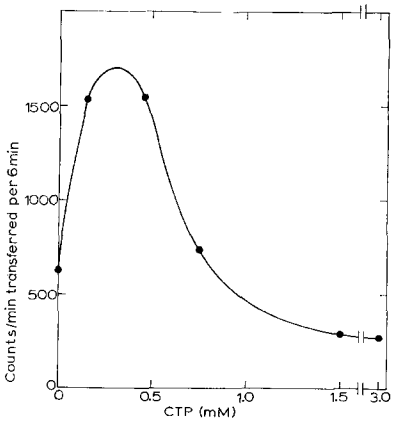
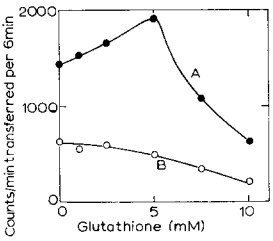


Fig. 3. Time course of NANA transfer. Incubation mixtures, as described under *Assay of NANA transfer*, are incubated for the times indicated.

Fig. 4. Effect of CTP concentration on the rate of NANA transfer. Incubation mixtures are as described under *Assay of NANA transfer* with the indicated CTP concentrations, and the omission of glutathione.



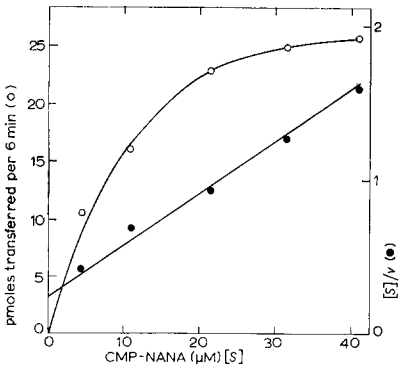


Fig. 5. Effect of glutathione concentration on the rate of NANA transfer. Incubation mixtures are as described under *Assay of NANA transfer* with the indicated concentrations of glutathione and the following concentrations of CTP: ●—●, 0.3 mM; ○—○, none.

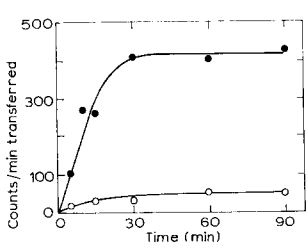
Fig. 6. Effect of CMP–NANA concentration on the rate of NANA transfer. Incubation mixtures are as descrined under *Assay of NANA transfer* with the indicated concentrations of CMP–[^{3}H]-NANA (22 000 counts/min per nmole). Each point is corrected for a zero-time control at the appropriate concentration of CMP–[^{3}H]NANA. The K_m for CMP–NANA is 7 μM.

acting to regenerate CMP–NANA hydrolyzed during the incubation. The synthetase from hog submaxillary gland exhibits a K_m of 0.6 mM for CTP and is also stimulated by glutathione. Higher concentrations of CTP might be expected to compete with CMP–NANA for the sialyl transferase as Fig. 4 indicates. Resuspension of the enzyme preparation in fresh homogenizing medium and re-centrifugation does not diminish the stimulatory effects of CTP and glutathione.

The K_m for CMP–NANA is 7 μM (Fig. 6).

Properties of UDP–galactose glycoprotein galactosyl transferase

The transfer of galactose is complete in about 15 min and shows an almost absolute requirement for Mn^{2+} (Fig. 7)[1]. The maximum rate of transfer is reached at a Mn^{2+} concentration of 2.5 to 5 mM (Fig. 8). UTP was shown previously[1] to stimulate galactose transfer in a 3-h incubation and has been routinely included. However, no



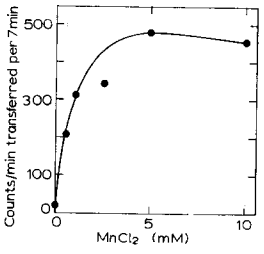


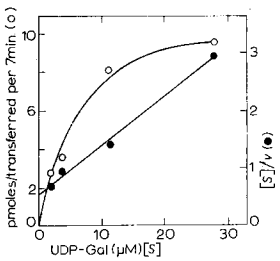
Fig. 7. Time course of galactose transfer. Incubation mixtures, as described under *Assay of galactose transfer*, contained the following concentrations of $MnCl_2$: ●—●, 2.5 mM; ○—○, none. Incubation times are as indicated.

Fig. 8. Effect of $MnCl_2$ concentration on the rate of galactose transfer. Incubation mixtures are as indicated under *Assay of galactose transfer* with the indicated concentrations of $MnCl_2$.

detailed study of its effect has been attempted. Presumably it interferes with the hydrolysis of UDP-galactose or regenerates it after hydrolysis. The K_m for UDP-galactose is 6.5 μM (Fig. 9).

UDP-Galactose-stimulated NANA transfer

As previously reported[1] the transfer of galactose residues to glycoprotein acceptor molecules provides additional acceptor sites for NANA transfer. The rapid transfer of galactose has the effect of prolonging the initial rapid NANA transfer.



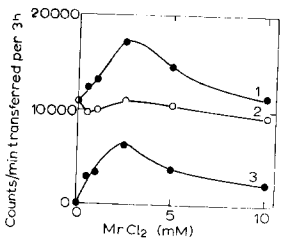


Fig. 9. Effect of UDP-galactose concentration on the rate of galactose transfer. Incubation mixtures, as described under *Assay of galactose transfer*, contain the indicated concentrations of UDP-[^{14}C]galactose (39 300 counts/min per mole). Each point is corrected for a zero-time control at the appropriate concentration of UDP-[^{14}C]galactose. The K_m for UDP-galactose is 6.5 μM.

Fig. 10. Effect of $MnCl_2$ concentration on UDP-galactose-stimulated NANA transfer. Incubation mixtures, as described under *Assay of NANA transfer*, contained the indicated $MnCl_2$ concentrations and the following UDP-galactose concentrations: Curve 1, 0.2 mM; Curve 2, none. Curve 3 is the difference between Curves 1 and 2.

The Mn^{2+} concentration required for maximum UDP-galactose stimulation is 2.5 mM (Fig. 10).

Both galactose transfer and UDP-galactose-stimulated NANA transfer are maximal at the same Mn^{2+} concentration, a further indication of the sequential transfer of galactose and NANA.

DISCUSSION

The particulate enzyme-acceptor system represents a highly efficient organization of enzyme and substrate which eliminates the need for random complex formation which occurs with soluble systems. Efficiency is also reflected in the low K_m values observed for the sugar nucleotide donors. The K_m values reported for CMP-NANA[5] and UDP-galactose[6] in wholly soluble transfer systems are 70-fold and 40-fold greater, respectively, than those reported here. Low K_m values would permit active glycoprotein synthesis to proceed with very low levels of donors. In rat liver, a microsomal NANA transfer system involved in the glycosylation of plasma proteins exhibits a K_m of 2 μM for CMP-NANA[7]. Although the enzymes are present which synthesize CMP-NANA[4,8-10], it has not been possible to detect this sugar nucleotide in the liver. The evidence available indicates that the amounts of NANA or NANA-containing nucleotides present in rat liver are very small[11]. Consequently a high degree of efficiency is necessary. This can be achieved with low K_m values for substrates, as

discussed above, or by compartmentalized pools of substrates. The particle-bond acceptors constitute compartmentalized pools in both liver and retina systems. Furthermore, there is some evidence that CMP–NANA synthetase is particle-bound, at least in the retina. The bulk of this enzyme in the retina can be isolated in a particulate fraction[12]. CTP stimulates the transfer of NANA and has also been shown to mediate the incorporation of free NANA in the absence of CMP–NANA[1]. Washing the retinal preparation does not diminish the CTP stimulation. As a result, CMP–NANA as well as acceptor may be compartmentalized in the sense that it may be synthesized at the site of NANA transfer.

The products of these glycosyl transfer reactions appear to be tightly bound to the particulate enzyme preparation[1] and may be structural components of the cells from which they are derived. Given the complexity of the retina, it is not possible at present to assign these enzymes to a particular cell type. Further studies on this problem are in progress.

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