FOLIC ACID EFFECTS ON GLYCOPROTEIN-GALACTOSYLTRANSFERASE: A RE-ASSESSMENT

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Summary Previous studies (1) have reported that folic acid increases the activity of several glycosyltransferases, including a galactosyltransferase of rat liver and kidney suggesting that folic acid may be involved in the control of glycoprotein biosynthesis. The present study provides an explanation for the previous results using a galactosyltransferase which transfers galactose to both a fetuin acceptor and N-acetylglucosamine. If the UDP-galactose is 1.5 μM in the assay there is stimulation by 1.4 mM folic acid or 5 mM 5'-AMP but there is not stimulation when the UDP-galactose is 0.6 mM. Crude homogenates contain a nucleotide pyrophosphate and phosphatase which liberate galactose from UDP-galactose. Both folic acid and 5'-AMP inhibit the nucleotide pyrophosphatase which protects the substrate and this becomes significant when the UDP-galactose concentration is low. Purified galactosyltransferase is inhibited by both folic acid and 5'-AMP. The observed stimulation of galactosyltransferase in crude systems by folic acid or AMP is due to substrate protection and it is therefore unlikely that folic acid is involved in the control of glycoprotein synthesis.

Glycoprotein glycosyltransferases in mammalian systems transfer a glycose from an appropriate nucleotide-sugar to an acceptor which is usually an incomplete carbohydrate side-chain of a glycoprotein or glycolipid:

$$\text{Nucleotide-glycose} + \text{Acceptor} \xrightarrow{M^{2+}} \text{glycose-Acceptor} + \text{nucleotide}$$

In most tissues the glycosyltransferases are particulate though some occur in soluble form in such body fluids as blood and milk (2). Kirschbaum and Bosmann (1) reported that folic acid increased the activity of a collagen : glucosyltransferase and fetuin : galactosyltransferase in crude rat liver and kidney preparations and suggested that folic acid may play a key role in glycoprotein synthesis. As much as a 50-fold increase was observed with the fetuin : galactosyltransferase (3) after chromatography of a 10,000 x g rat kidney homogenate through Sephadex G-75 and Bio-Gel P-200 columns. Furthermore (4) it was reported that lysolecithin as well as folic acid stimulated the fetuin : galactosyltransferase. Various explanations were offered for the

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observed stimulation by folic acid, folic acid analogs and lysolecithin as related to possible control of the glycosyltransferases.

This laboratory has studied extensively the kinetic and physical properties of a soluble galactosyltransferase (2) which transfers galactose to glucose in the presence of α-lactalbumin and in its absence will transfer galactose to a non-reducing GlcNAc on β-glycosides, including fetuin which has had NANA and galactose removed (acceptor used in 1, 2, 4). That is, the crude and non-solubilized enzyme used by Kirschbaum and Bosmann (1, 2, 4) is probably identical to the highly purified enzyme isolated from milk (2). Preliminary experiments showed that purified galactosyltransferase was not activated by folic acid or lysolecithin but rather folic acid was slightly inhibitory. This paper presents data that provides an explanation for the apparent activation of the galactosyltransferase by folic acid. The results show that folic acid is a good inhibitor of UDP-galactose hydrolysis and probably inhibits nucleotide pyrophosphatase. In the absence of nucleotide pyrophosphatase inhibitors such as folic acid or 5'-AMP, the pyrophosphatase will greatly reduce the concentration of UDP-galactose. It is unfortunate that the previous experiments (1, 2, 4) were done with UDP-galactose in the μM range by only using UDP-galactose-^{14}C. If the UDP-galactose concentration is raised to the mM range, no stimulation of galactosyltransferase by folic acid is observed in rat kidney homogenates. Many assays for glycosyltransferases rely upon measuring the incorporation of the glycose into a carbohydrate-protein acceptor which is then precipitated by acid and counted. As a result hydrolysis of UDP-galactose due to the other enzymes is not measured in a control experiment and this can lead to erroneous conclusions especially when the nucleotide-sugar concentration is low.

Materials and Methods

Fetuin (minus sialic acid and galactose) was a gift from Dr. E. H. Eylar. Ovalbumin, folic acid, 2-amino-4-hydroxypteridine-6-carboxylic acid, lysolecithin and 5'-AMP were from Sigma and UDP- ^{14}C galactose (240 Ci/mole)

was from New England Nuclear Corp. Bio Gel P-60 was from Bio Rad. Galactosyltransferase from bovine milk was purified (5) and assayed for 20 minutes as previously described (6) using GlcNAc as the galactosyl acceptor. In the absence of the galactosyl acceptor, the assay measures the hydrolysis of UDP-galactose to free galactose. This reaction occurs by the action of a nucleotide pyrophosphatase (7-9) to form UMP and Gal-1-P which is then hydrolyzed to galactose by a phosphatase. The overall reaction due to the two enzymes will be referred to as UDP-galactose "hydrolase." Fetuin at 240 µg or ovalbumin at 0.8 mg per 100 µl of final assay volume are also galactosyl acceptors and were acid precipitated and counted as described in (1). Galactosyltransferase was also assayed under the conditions described by Kirschbaum and Bosmann (1) using fetuin as the galactosylacceptor and only ^{14}C-UDP-galactose so that the final assay concentration was 1.5 µM. This assay does not measure the hydrolysis of UDP-galactose since the product of the reaction is precipitated with acid and counted. Homogenates from adult male Fischer rat kidneys were prepared as described (1) or by homogenizing in a Polytron, setting 4 for 20 seconds at 4°. Five ml of a 10,000 x g supernatent of rat kidney was chromatographed on a 2.5 x 33 cm Bio Gel P-60 column at 4°, equilibrated and eluted with 0.1 M Tris - 0.1% Triton X-100 - 2 mM mercapethanol, pH 6.8. Three ml fractions were collected and the first three turbid tubes at the void volume were pooled and were either stored frozen or at 4°.

Results

Homogenous galactosyltransferase from bovine milk does not hydrolyze UDP-galactose but does transfer galactose to the fetuin acceptor (-NANA,-Gal). It was not stimulated by folic acid but rather was inhibited. 1.4 mM folic acid inhibited the bovine milk galactosyltransferase under standard assay conditions (6) by about 10 percent as did mM 5'-AMP; 5 mM 5'-AMP inhibited 25 percent as did 1.4 mM 2-amino-4-hydroxypteridine-6-carboxylic acid, an analog of the pterol group in folic acid.

Lactating rat mammary gland is an excellent source of particulate galactosyltransferase (10). Assayed under our conditions (6) for the transfer of galactose to GlcNAc preliminary experiments indicated no effect of 1.4 mM folic acid in a variety of crude mammary gland fractions except that the hydrolysis of UDP-galactose was reduced by 60 percent. Assayed under the Kirschbaum and Bosmann (1) conditions (1.5 µM UDP-galactose) with fetuin (-NANA and galactose) about a two fold enhancement was observed by 1.4 mM folic acid but no enhancement was observed when the UDP-galactose was 0.6 mM. Both folic acid and 5'-AMP inhibited the hydrolysis of UDP-galactose . An examination of the data showed that the degree of enhancement by folic acid or

5'-AMP (with low UDP-galactose) correlated with the amount of UDP-galactose "hydrolase" in the preparations.

Attempts were made to repeat the experiments described by Kirschbaum and Bosmann (1, 2, 4) utilizing rat kidney preparations. The results presented in Table 1 show that folic acid gives a 3-4 fold activation of galactosyl-

Table 1

Effect of Folic Acid on Rat Kidney Galactosyltransferase

Galactosyl Acceptor	Additions	Units/ml
Fetuin[1]	None	0.0062
Fetuin[1]	1.4 mM folic acid	0.020
Fetuin[2]	None	0.59
Fetuin[2]	1.4 mM folic acid	0.59
Fetuin[3]	None	1.50
Fetuin[3]	1.4 mM folic acid	1.31
Fetuin[4]	None	0
Fetuin[4]	1.4 mM folic acid	0.27

Twenty µl of extract from a 10,000 x g fraction of rat kidneys homogenized in a Polytron was assayed for galactosyltransferase activity with various acceptors. Similar results were obtained with 40 µl of extract except that the fold activation with fetuin[1] was 4.1 instead of 3.2 as reported in the Table. Units refers to the µmoles of galactose incorporated per min at 37°. The UDP-galctose "hydrolase" activity was 2092 cpm/20 µl extract which represent 20% of the total counts and this was inhibited 70% by 1.4 mM folic acid.

[1]UDP-galactose was 1.5 µM and assayed as described (1)
[2]UDP-galactose was made to 0.6 mM and assayed as described (1)
[3]UDP-galactose is 0.6 mM and assayed as previously (6)
[4]UDP-galactose is 0.6 mM and GlcNAc is the acceptor and assayed as previously (6).

transferase when assayed in the presence of 1.5 µM UDP-galactose (1) but when the UDP-galactose was increased to 0.6 mM, no apparent activation by folic acid was observed. No activation, but rather a slight inhibition was observed with folic acid when assayed under our standard conditions with the fetuin galactosylacceptor. No activity was observed when GlcNAc was the galactosyl acceptor unles folic acid was present which was due to an 80% inhibition of

the UDP-galactose "Hydrolase." These data show that the apparent activation of galactosyltransferase was due to an inadequate amount of UDP-galactose in the assay and the competing enzymes which hydrolyze UDP-galactose.

Kirschbaum and Bosmann (1) also reported that lysolecithin enhanced the rate of galactosyltransferase by about two fold. We have had difficulty in repeating such experiments though the data presented in Table 2 indicates a

Table 2

Apparent Enhancement of Rat Kidney Galactosyltransferase

	Units/ml x 10^3	
Additions	1.5 μM UDP-galactose	0.6 mM UDP-galactose
None	8.5	6.5
Folic Acid, 1.4 mM	21.6	6.9
Folic Acid, 0.7 mM	26.2	7.7
5'-AMP, 5 mM	24.6	6.9
Lysolecithin, 60 μg/ml	8.5	8.9
Folic (0.7mM) + lysolecithin (60 μg/ml)	37.7	6.9
5'-AMP (5 mM) + lysolecithin (60 μg/ml)	39.2	7.7

An extract of rat kidney was prepared as described (1) and the supernatent solution (3 ml) from a 10,000 x g centrifugation was passed over a P-60 column (Methods) and the first three tubes at the void volumn were pooled for assay. Assays were as described (1, 4) with fetuin as the acceptor and the UDP-galactose at 1.5 μM or made to 0.6 mM.

synergistic effect when lysolecithin is included with 0.7 mM folic acid or 5 mM 5'-AMP when the UDP-galactose concentration is 1.5 μM. 5'-AMP is as effective as folic acid in activating the galactosyltransferase when the UDP-galactose concentration is 1.5 μM but no effect is observed at 0.6 mM UDP-galactose. Ovalbumin is also a substrate for the galactosyltransferase (1) and there was activation by folic acid when it replaced fetuin and when the UDP-galactose was 1.5 μM.

Some properties of the UDP-galactose "hydrolase" were examined in crude rat kidney preparations. The assay was linear with respect to crude extract from 0 to 50% incorporation when the UDP-galactose was 0.6 mM. The apparent K_m for UDP-galactose was between 0.3 to 0.5 mM which is comparable to the K_m for UDP-galactose by a highly purified nucleotide pyrophosphatase isolated from rat liver plasma membrane (7). 5'-AMP is an effective inhibitor of this liver enzyme (7, 9) and the K_i is 0.26 mM. Inhibition studies with the rat kidney 10,000 x g supernatant preparations gave the following percent inhibition of UDP-galactose hydrolysis when UDP-galactose was 0.6 mM: 0.7 mM folic acid, 74.1%; 1.0 mM 5'-AMP, 47.5%; 5.0 mM 5'-AMP, 95.3%; 2-amino-4-hydroxypteridine-6-carboxylic acid at 0.7 and 1.4 mM was 34.6% and 59.8% respectively. Lysolecithin at 60 and 120 µg/ml had no affect on UDP-galactose hydrolysis. Both 5'-AMP and folic acid are good inhibitors and it appears that the pteroyl portion of folic acid which may be considered a structure analog of the adenine portion of AMP is responsible for the inhibitory properties of folic acid and its derivatives.

Discussion

Kirschbaum and Bosmann (1, 2, 4) reported that folic acid and lysolecithin enhance the activities of glycosyltransferases such as a glucosyl (to collagen) and galactosyl (to fetuin) but inhibited NANA transfer to fetuin (-NANA). They suggested that folic acid may play a key role in regulating glycoprotein synthesis. The present experiments indicate that folic acid inhibits a nucleotide pyrophosphatase which cleaves the nucleotide sugar to a 5'-nucleotide and a sugar phosphate which is subsequently hydrolyzed to the free sugar by a phosphatase and as a result protects the substrate for the glycosyltransferase. Increasing the UDP-galactose from 1.5 µM to 0.6 mM in the assay obliterates the folic acid effect. 5'-AMP, a good inhibitor of nucleotide pyrophosphatase (7) can mimic folic acid enhancement at low UDP-galactose concentrations. Folic acid, 5'-AMP and 2-amino-4-hydroxypteridine-

6-carboxylic acid slightly inhibit highly purified galactosyltransferase isolated from bovine milk as well as markedly inhibiting the nucleotide pyrophosphatase. Nucleotide pyrophosphatase (7) has been solubilized from rat liver plasma membranes by 0.2% Triton X-100 and pancreatic lipase and purified to apparent homogeniety. The enzyme hydrolyzes UDP-glucose, UTP and ATP with about the same K_m (0.3 - 0.8 mM) but the maximum velocities differ. For UDP-glucose, the maximum velocity is 120 μmoles/min/mg at 25° and this should be comparable for UDP-galactose whereas the apparent V_{max} for the purified galactosyltransferase is between 1-2 μmoles /min/mg at 20° (1). A similar enzyme from hamster embryo cell cultures particulate fractions hydrolyzes UDP-N-acetyl-D-galactosamine and UDP-N-acetyl-D-glucosamine (8). The enzyme prefers nucleotides with pyrophosphate bonds as substrates and would not be expected to hydrolyze CMP-NANA readily and thereby explaining why Kirschbaum and Bosmann (1) did not observe an enhancement with a sialyltransferase. Both the nucleotide pyrophosphatase and galactosyltransferase are membrane bound. Lysolecithin had no effect on the nucleotide pyrophosphatase but may have partially activated the membrane bound galactosyltransfer. Previous studies (1) have shown that low amounts of detergents increase the activity of membrane bound galactosyltransferase. No apparent enhancement of galactosyltransferase activity was observed in various rat mammary gland particulate preparations which are a good source of galactosyltransferase. These preparations had relatively low levels of "hydrolase" when compared to the kidney preparations. It also became evident from this study that the amount of activation observed at low UDP-galactose was dependent upon the ratio of the galactosyltransferase and "hydrolase" and the absolute amount of both enzymes. The two enzymes appear to differ greatly (100 fold) in V_{max} though their K_m for UDP-galactose is comparable which means that the nucleotide pyrophosphatase can utilize UDP-galactose about 100 times more rapidly than the galactosyltransferase. Homogenizing and storage procedures will influence the apparent enhancement with folic acid or 5'-AMP at low UDP-galactose. The nucleotide

pyrophosphatase was more stable than the galactosyltransferase and aged preparations stored at 4° or frozen gave less enhancement which may be an explanation in part for our difficulty in achieving the enhancements observed by Kirschbaum and Bosmann (1, 2, 4).

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