Biochimica et Biophysica Acta, 523 (1978) 53—58

BBA 68379

IMMOBILIZED BOVINE LACTOSE SYNTHASE

A METHOD OF TOPOGRAPHICAL ANALYSIS OF THE ACTIVE SITE

JACOB GRUNWALD * and LAWRENCE J. BERLINER **

Department of Chemistry, The Ohio State University, Columbus, Ohio 43210 (U.S.A.)

(Received August 10th, 1977)

Summary

Bovine galactosyltransferase (UDPgalactose : D-glucose 4β-galactosyltransferase, EC 2.4.1.22) was covalently coupled to Sepharose 4B by reaction at pH 5.0 with the activated mixed disulfide Sepharose-glutathione-2(5-nitropyridyl)-disulfide. The Sepharose-protein conjugate was presumably coupled via the unique highly reactive cysteine of those thiols on the bovine enzyme. The gel-bound *N*-acetyllactosamine and lactose synthase activity of about 0.4% was consistent with the effects of diffusion and the 90% activity reduction noted upon thiol modification of the dissolved enzyme.

The residual lactose biosynthetic activity of the bound enzyme appeared possible only if the reactive thiol were physically distinct from the active site since the bulky Sepharose-glutathione group must not obscure the α-lactalbumin binding region.

Introduction

Galactosyltransferase (UDPgalactose : D-glucose 4β-galactosyltransferase, EC 2.4.1.22) of bovine milk catalyzes the two basic reactions:

$$\text{UDPGal} + \text{GlcnNAc} \xrightarrow{\text{A,Mn(II)}} \text{UDP} + \textit{N}\text{-acetyllactosamine} \quad (1)$$

$$\text{UDPGal} + \text{Glucose} \xrightarrow{\text{A,B,Mn(II)}} \text{UDP} + \text{lactose} \quad (2)$$

* Present address: Artificial Organ Research Unit, Department of Physiology, McGill University, School of Medicine, Montreal, Quebec, H3C 3G1, Canada.

** To whom correspondence should be addressed.

Abbreviations used: UDPGal and UDPGlc, uridine diphosphate-galactose and -glucose, respectively; GlcnNAc, *N*-acetylglucosamine.

where A is galactosyltransferase and B is the modifier protein, α-lactalbumin, which enhances lactose biosynthesis where glucose is the acceptor (Reaction 2). While the primary structure of galactosyltransferase is not yet known, a great deal of information is potentially available from examination of its molecular properties.

Magee and Ebner [1] showed that *N*-ethylmaleimide or *p*-chloromercuribenzoate reduced galactosyltransferase activity to approx. 10% of that for native enzyme while not totally inactivating the enzyme. Since the K_m for UDPGal was unchanged they concluded that the reduction in activity was reflected in a 90% reduction in the catalytic rate constant. While UDP or UDPGal (especially in the presence of Mn(II)) significantly reduced the rate of inactivation, this "substrate protection" effect was probably elicited by a structural change between the two distinct sites since the unaltered K_m for UDPGal suggested that the UDPGal and thiol sites were not coincidental. A further complication which was not addressed earlier [1] arises from the fact that at least one additional thiol group exists in the bovine milk enzyme [2]. Such was not the case with the single thiol-containing human enzyme where similar results were obtained [3].

While attempting to develop efficient high scale isolation procedures for galactosyltransferase we investigated the efficacy of thiol-Sepharose covalent chromatography as used eloquently by Brocklehurst et al. [4,5] for the purification of papain. We report here some observations on the lactose synthase activity of the bound enzyme and speculations on active site topography.

Experimental Procedure

Materials. UDPGal, phospho*enol*pyruvate, NADH, ATP, dithiothreitol and glutathione were from Sigma Chemical Co. Dithiobis-nitrobenzoic acid or Ellman's reagent was from ICN-NBC. Sepharose 4B was purchased from Pharmacia, and 2,2′-dithiobis-5-nitropyridine was from Aldrich.

Proteins. Pyruvate kinase (type 1) and α-lactalbumin were from Sigma Chemical Co. Galactosyltransferase was purified by the methods of Geren et al. [6] through the norleucine-Sepharose hydrophobic chromatography step but was then followed by one or two steps with α-lactalbumin-Sepharose to yield a highly pure enzyme of specific activity typically at 18—20 units/mg at 30°C. The highly pure enzyme was not, however, separated into its individual molecular weight forms [7].

Affinity chromatography. Glutathione-Sepharose 4B was prepared after the method of Brocklehurst et al. [5]. The column was activated with a 50% dioxane solution of the chromophoric group 2,2′-dithiobis-5-nitropyridine which was advantageous by its intense yellow color; $\epsilon_{386nm} = 1.4 \cdot 10^4\ M^{-1} \cdot cm^{-1}$ [8].

In a typical experiment 10—20 units of highly pure galactosyltransferase were coupled to 1 ml of swollen activated gel in a centrifuge tube at room temperature, pH 5.0—5.3, 0.1 M sodium acetate (degassed), 1 mM EDTA, 1 mM ϵ-aminocaproic acid, for 30 min. After washing several times with 0.05 M Tris · HCl, pH 8.0, containing 1 mM ϵ-aminocaproic acid, the pure enzyme was released from the gel by treating with 20 mM dithiothreitol in the same buffer for 30 min or less. Thiol groups were measured with dithiobis-nitrobenzoic acid

in the presence or absence of 8 M urea [3].

Enzyme assays. Soluble galactosyltransferase activity was measured by either the spectrophotometric coupled assay of Fitzgerald et al. [9] or the radiometric assay of Trayer and Hill [10]. Insolubilized enzyme was measured either by the above two assays with frequent agitation of the assay vials, or by a "UDP generation assay" where timed aliquots were taken from a constantly stirred solution of gel-bound enzyme, $MnCl_2$, UDPGal, GlcnNAc or glucose and α-lactalbumin, and assayed for UDP with the coupling enzyme mix used in the normal spectrophotometric assay [9].

In order to account for any "leakage" of bound enzyme into the supernatant, a suspension of insolubilized enzyme in buffer was also assayed with time for soluble enzyme activity.

Results

Covalent chromatography. Fig. 1 depicts the coupling scheme utilized herein. The gel capacity was 3—10 μmol/ml by thiol analysis. Table I lists

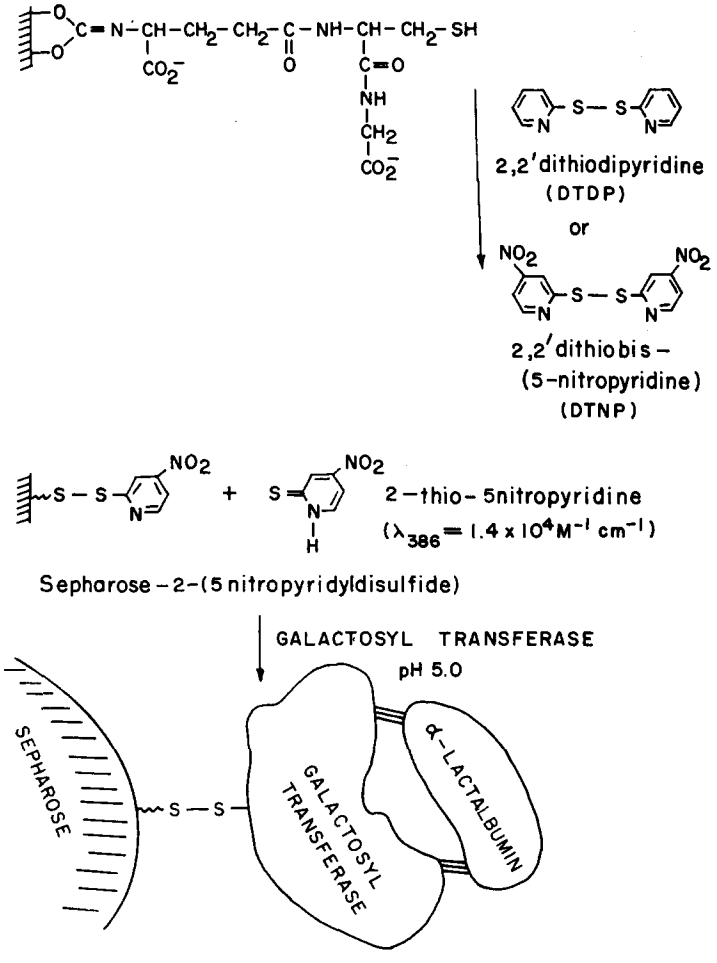

Fig. 1. A protein coupling to thiol (glutathione)-Sepharose.

TABLE I

GALACTOSYL TRANSFERASE BINDING TO GLUTATHIONE SEPHAROSE

Activated thiol-Sepharose and enzyme were gently mixed at the indicated pH and temperature in degassed (0.1 M) acetate or (0.05 M) Tris · HCl buffer, with 1 mM EDTA and 1 mM ε-aminocaproic acid. Aliquots were removed with time and assayed for Reaction 1.

pH, Temperature	Time (min)	Activity remaining in the supernatant (%)
7.4, 25°C	0	100
	10	100
	120	83.4
5.5, 4°C	0	100
	720	66.8
5.1, 25°C	0	100
	10	0

approximate coupling times and extent of galactosyltransferase removal from the supernatant when exposing a highly active protein solution to approx. 1 g of swollen gel at pH values varying from 5.0 to 7.4. Surprisingly, the coupling was extremely slow near the catalytic pH optimum (pH 7.4) yet quite rapid ($t_{1/2} \approx 5$—15 min) around pH 5.0, as we have found with other thiol reagents as well.

The efficiency of coupling and removal approached 100% for up to 20 units of purified enzyme; however, attempts with this enzyme at earlier stages of purification did not give 100% recovery, nor, unfortunately, did the purification factor warrant its incorporation into a routine isolation scheme (see Table II) where factors of many hundred fold are required.

Activity of bound enzyme. Highly pure galactosyltransferase (spec. act. 18—20 units/mg) coupled to glutathione-Sepharose was assayed for *N*-acetyllactosamine (Reaction 1) and lactose synthase (Reaction 2) activity by all three methods described in Experimental Procedure. We were particularly cautious

TABLE II

PURIFICATION WITH GLUTATHIONE-SEPHAROSE

Examples for two samples previously purified through the norleucine-Sepharose hydrophobic chromatography step. All experiments are for approx. 1 ml of swollen gel. Coupling to the gel was performed at pH 5.0—5.3; removal with dithiothreitol (in 0.05 M Tris · HCl, 1 mM EDTA and 1 mM ε-aminocaproic acid) was performed at pH 8.0. Enzyme activity was measured for Reaction 1 as in Table I for the coupling step (binding supernatant) and after addition of dithiothreitol (removing supernatant) for the uncoupling from the gel.

	Specific activity (units/mg)	Total activity (units)	Yield (%)	Purification factor
Sample No. 1	0.09	0.97	—	—
Binding supernatant	—	0	—	—
Removing supernatant	0.45	0.89	92	5.0
Sample No. 2	0.15	22.2	—	—
Binding supernatant	—	1.4	—	—
Removing supernatant	5.7	14.5	65	38

TABLE III

ACTIVITY OF IMMOBILIZED GALACTOSYLTRANSFERASE

Gel-bound enzyme was assayed by both the spectrophotometric [9] and radiometric [10] methods for *N*-acetyllactosamine or lactose biosynthesis, Reactions 1 or 2, respectively. Approx. 0.05 ml of gel was assayed each time. Released enzyme activity was that determined from the gel supernatant after treatment with dithiothreitol under conditions stated in Table II. *N*-Ethylmaleimide modification was performed by mixing 1 ml of gel-bound enzyme in 5 ml of 10 mM *N*-ethylmaleimide/0.05 M Tris · HCl (pH 7.4)/0.1 M $(NH_4)_2SO_4$ for 1 h at ambient temperature followed by extensive washing with the same buffer. Leakage activity was measured by the spectrophotometric or UDP generation methods as described in Experimental Procedure. The relative amount of enzyme leakage was of the order of approx. 20% of the observed gel bound activity. Leakage from the *N*-ethylmaleimide-treated gel-bound enzyme was usually higher (up to 45% of the observed gel bound activity).

Assay method	Relative activity (immobilized/released, %)	
	"Native" enzyme	After *N*-ethylameimide treatment
Reaction 1		
Spectrophotometric or radiometric	0.42	0.20
UDP generation	0.33	n.d.
Reaction 2		
Spectrophotometric or radiometric	0.39	0.18—0.22
UDP generation	0.30	n.d.

n.d., not determined.

to account for any "leakage activity" caused by slowly released, unbound enzyme present in the gel mixture. The results measured for a typical gel sample which was also further modified with *N*-ethylmaleimide are listed in Table III. These rates, while relatively small compared with soluble enzyme, were finite and measurable.

Discussion

The results presented above clearly display both *N*-acetyllactosamine and lactose synthase activity on immobilized thiol-bound galactosyltransferase. The measured residual activities of 0.2—0.4% were quite reasonable after considering the 5—10% residual activity of thiol-blocked soluble enzyme [7] and factors commonly in the range of 1/10—1/20 for diffusional and other effects encountered in immobilized or crystalline systems [11]. The comparable efficacy of galactosyltransferase activity with α-lactalbumin (lactose synthase) suggests that the site of this quite bulky modification (glutathione-Sepharose) must be at a thiol group sufficiently removed from the α-lactalbumin binding site. While the α-lactalbumin binding site and the substrate binding site may not necessarily coincide (as depicted in Fig. 1) the results from this and previous work place the reactive thiol(s) away from the substrate and/or α-lactalbumin site(s) [7]. One ambiguity remains in this work which may be difficult if not impossible to rule out completely. Since there are probably two thiols per average milk enzyme species (molecular weights 55 000—58 000 and 42 000—45 000) we cannot dismiss the possibility of gel coupling to, e.g. 90% of the highly reactive, active site linked, sensitive thiol and approx. 10% of the second

less sensitive thiol, leaving 10% of the bound enzyme with the sensitive thiol unblocked. The gel-bound enzyme, after modification with *N*-ethylmaleimide (see Table III), displayed 50% of its previous activity (and 50% of the native enzyme activity when released with dithiothreitol) *. This would suggest that, with all thiols blocked, the gel-bound enzyme still retained activity, consistent with coupling through the sensitive thiol, the less sensitive thiol eliciting an approx. 50% rate reduction effect on enzyme activity. One other result which suggests coupling through the sensitive thiol results from an experiment at pH 5.1 where it was shown that Mn(II)-UDPGal (4 mM : 2 mM) offered "substrate protection" to gel coupling by slowing the rate of gel coupling, conditions where the rate of *N*-ethylmaleimide inactivation of dissolved enzyme was also reduced [1]. No protection was observed with α-lactalbumin (0.4 mM) and GlcnNAc (18 mM) offering further support to the topographical model of the reactive thiol locus.

Acknowledgements

This work was supported in part by a grant from the U.S.P.H.S. (GM 21923). We thank Dr. Piet Jan Andree for many helpful discussions. L.J.B. is an Established Investigator of the American Heart Association

References

1 Magee, S.C. and Ebner, K.E. (1974) J. Biol. Chem. 249, 6992—6998
2 Powell, J.T. and Brew, K. (1974) Eur. J. Biochem. 48, 217—228
3 Kitchen, B.J. and Andrews, P. (1974) Biochem. J. 141, 173—178
4 Brocklehurst, K., Carlsson, J., Kierstan, M.P.J. and Crook, E.M. (1973) Biochem. J. 133, 573—584
5 Brocklehurst, K., Carlsson, J., Kierstan, M.P.J. and Crook, E.M. (1974) Methods Enzymol. 34B, 531—544
6 Geren, C.R., Magee, S.C. and Ebner, K.E. (1976) Arch. Biochem. Biophys. 172, 149—155
7 Magee, S.C., Mawal, R. and Ebner, K.E. (1974) Biochemistry 13, 99—102
8 Grassetti, D.R. and Murray, Jr., J.F. (1969) J. Chromatogr. 41, 121—123
9 Fitzgerald, D.K., Colvin, B., Mawal, R. and Ebner, K.E. (1970) Anal. Biochem. 36, 43—61
10 Trayer, I.P. and Hill, R.L. (1971) J. Biol. Chem. 246, 6666—6675
11 Katchalski, E., Silman, I. and Goldman, R. (1971) Adv. Enzymol. 34, 445—536

* Enzyme which was previously treated at pH 5.1 with 0.25 mM dithiobis-nitrobenzoic acid and dialyzed to yield a 1 : 1 (mol/mol) blocked, 10% active enzyme apparently coupled to the activated gel; however, the possibility of a mixed disulfide interchange cannot be ruled out here.