A comparison of the active site of maltase–glucoamylase from the brush border of rabbit small intestine and kidney by chemical modification studies

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The neutral maltase–glucoamylase complex has been purified to homogeneity from the brush-border membrane of rabbit intestine and kidney. Chemical modification of the amino acid side chains was carried out on the purified enzymes. Studies on the kidney enzyme revealed that tryptophan, histidine and cysteine were essential for both maltase and glucoamylase activities, whereas tryptophan, histidine and lysine were essential for the maltase and glucoamylase activities of the intestinal enzyme. Though there was no difference in the amino acids essential for the hydrolysis of maltose and starch by any one enzyme, starch hydrolysis seems to require two histidine residues instead of the one which is required for maltose hydrolysis. This appears to be true for both the intestinal and kidney enzymes.

INTRODUCTION

The brush borders of the small intestine and kidney, besides possessing histological similarity, are also endowed with a similar complement of enzymes. Among the glycosidases, maltase–glucoamylase and trehalase are present in the intestine as well as the kidney brush border, whereas sucrase is present exclusively in the intestine. Maltase and glucoamylase activities are known to exist in the form of a complex which has the highest molecular mass among the glycosidases (Semenza, 1986). The neutral maltase–glucoamylase has been purified from intestine (Sivakami & Radhakrishnan, 1973, 1976) and kidney (Pereira & Sivakami, 1989) of the rabbit, and some of the properties have been compared. They seem to have similar substrate specificity but differ in quaternary structure (Pereira & Sivakami, 1989).

Most of the earlier work on the glycosidases has emphasized the mode of anchoring of these membrane proteins (Semenza, 1986). There is little information on the amino acids present in the active site of these enzymes. The presence of certain amino acids in the active site has been indicated indirectly by kinetic studies such as variation of K_m and V_{max} with pH. The possible involvement of an imidazole group at the active site of the maltase–glucoamylase from rabbit intestine (Sivakami & Radhakrishnan, 1976), and of an imidazole or a thiol group at the active site of the renal maltase–glucoamylase (Pereira & Sivakami, 1989), has been indicated by these kinetic studies.

In the present paper we report the results of chemical modification experiments on the hydrolysis of maltose and starch by the maltase–glucoamylase complex from intestine and kidney of the rabbit.

MATERIALS AND METHODS

Chemicals

The following materials were purchased as indicated. Glucose oxidase, horseradish peroxidase, Trizma buffer, papain, octyl-

agarose, *N*-acetylimidazole, *N*-bromosuccinimide (NBS), 2-hydroxy-5-nitrobenzyl bromide (HNBB), pyridoxal phosphate, 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide, diethyl pyrocarbonate (DEP), *N*-ethylmaleimide (NEM) and *p*-hydroxymercuribenzoate (PHMB) were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Sephadex G-200 was from Pharmacia, Uppsala, Sweden. Tetranitromethane was from Spectrochem, Bombay, India. Folin–Ciocalteu reagent was from Sisco Laboratories, Bombay, India. Phenylglyoxal and 2,4,6-trinitrobenzenesulphonate (TNBS) were gifts from Professor A. Surolia, Indian Institute of Science, Bangalore, India. All other chemicals were of the highest analytical grade available.

Assay of enzyme activities

Maltase and glucoamylase activities were assayed by measurement of glucose released from maltose or starch by the Tris–glucose oxidase–peroxidase procedure of Dahlqvist (1964) as described previously (Pereira & Sivakami, 1989). Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Enzyme units

One unit of enzyme activity is defined as the amount of enzyme required to liberate 1 μ mol of glucose/min at 37 °C under standard assay conditions.

Purification of maltase–glucoamylase from intestine

Adult rabbits of either sex were killed, and intestines were removed and washed with 1.15% KCl, and the mucosa was scraped with a blunt knife. A homogenate of the mucosal scrapings was prepared in 0.01 M-potassium phosphate buffer, pH 7.0, containing 50 mM-mannitol.

Brush-border vesicles were prepared by the Ca^{2+} precipitation method of Ganapathy *et al.* (1981).

For solubilization of the membrane-bound activity, papain was added at a papain-protein/membrane-protein ratio of 1:100

Abbreviations used: NBS, *N*-bromosuccinimide; HNBB, 2-hydroxy-5-nitrobenzyl bromide; DEP, diethyl pyrocarbonate; NEM, *N*-ethylmaleimide; PHMB, *p*-hydroxymercuribenzoate; TNBS, 2,4,6-trinitrobenzenesulphonate.

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Table 1. Purification of rabbit renal maltase–glucoamylase complex on octyl-agarose

Abbreviations: M, maltase; G, glucoamylase.

Fraction	Total activity (units)		Specific activity (units/mg)		Yield (%)		Enrichment (fold)	
	M	G	M	G	M	G	M	G
Homogenate	840	735	1.4	1.24	100	100	1.0	1.0
Brush-border vesicles	540	540	17	17	64	73	12	14
Papain supernatant	408	328	47	37	49	45	34	30
Octyl-agarose eluate	240	198	400	330	29	27	286	266

Table 2. Experimental conditions for the chemical modification of intestinal and kidney enzymes

Abbreviation: EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide.

Specificity	Modifying reagent	Concn. (mM)	Potassium phosphate buffer	Temperature	Time (min)	Reference
Tyrosine	<i>N</i> -Acetylimidazole	10–200	20 mM, pH 7.0	37 °C	20	Riordan & Vallee (1972)
Tyrosine	Tetranitromethane	0.2–4.0	20 mM, pH 8.0	37 °C	30	Bunning <i>et al.</i> (1978)
Tryptophan	NBS	0.001–0.025	20 mM, pH 5.9	37 °C	20	Mannervik <i>et al.</i> (1975)
Tryptophan	HNBB	1.0–10.0	100 mM, pH 5.9	4 °C	3	Horton & Koshland (1972)
Lysine	TNBS	0.5–2.0	20 mM, pH 8.0	37 °C	30	Fields (1972)
Lysine	Pyridoxal phosphate	0.5–4.0	20 mM, pH 6.8	37 °C (dark)	30	Phillips <i>et al.</i> (1983)
Glutamate/aspartate	EDC	10–100	100 mM, pH 6.0	37 °C	60	Bond <i>et al.</i> (1981)
Histidine	DEP	0.25–10.0	20 mM, pH 7.0	37 °C	30	Burstein <i>et al.</i> (1974); Daron & Aull (1982)
Cysteine	NEM	1.0–20	20 mM, pH 7.0	37 °C	30	Riordan & Vallee (1972)
Cysteine	PHMB	0.2–2.0	20 mM, pH 8.0	37 °C	30	Riordan & Vallee (1972)
Arginine	Phenylglyoxal	1.0–4.0	50 mM, pH 7.0	37 °C	30	Manohar & Appaji Rao (1984)

and incubated at 37 °C for 60 min, and the mixture was centrifuged at 20000 *g* for 120 min in a Sorvall RC-5B refrigerated centrifuge. Almost all the maltase–glucoamylase was released into the supernatant. This was then purified to homogeneity by making use of the specific affinity of glucoamylase to Sephadex G-200 by a procedure similar to that reported previously (Sivakami & Radhakrishnan, 1973). The enzyme was homogeneous on polyacrylamide-gel electrophoresis, both native and in the presence of SDS (Laemmli, 1970). The specific activity of the purified enzyme matched well with that reported previously. This preparation was used for chemical modification.

Purification of maltase–glucoamylase from kidney

Adult rabbits of either sex were killed, and kidneys were removed and the cortices separated. Then 16 g of cortices were weighed, minced and homogenized in 0.01 M-sodium phosphate buffer, pH 7.0, containing 50 mM-mannitol. Brush-border vesicles were prepared by the calcium precipitation method of Ganapathy *et al.* (1981). For solubilization, papain was used exactly as described above. The supernatant, which contained maltase–glucoamylase activity, was purified by hydrophobic chromatography on an octyl-agarose column (bed volume 2.0 ml), equilibrated with 0.01 M-sodium phosphate buffer, pH 7.0. The solubilized extract was left in contact with the gel column for 1 h and then washed with the equilibrating buffer.

The break-through and washes contained no maltase–glucoamylase activity. Elution was carried out with 0.3 M-sodium phosphate buffer, which eluted maltase–glucoamylase specifically. This preparation was homogeneous on native and SDS/polyacrylamide-gel electrophoresis (Laemmli, 1970), as shown by Coomassie Blue and silver staining (Morrissey, 1981). A summary of the purification is shown in Table 1. This preparation was dialysed thoroughly against 0.01 M-potassium phosphate buffer, pH 7.0, and was used for chemical modification.

Chemical modification studies

Chemical modification of the enzyme was carried out by the addition of protein-modifying reagents to the pure enzyme (0.1 mg/ml) and incubating the mixture for the specific period of time as shown in Table 2. The buffers also varied with the reagents, as indicated in Table 2. At the end of the incubation, free amino acid (10 mM) was added in most cases to stop the reaction. When TNBS and pyridoxal phosphate were the reagents, 0.5 mM-Na₂SO₃ was included in the buffer as addition of free lysine resulted in high blanks. When PHMB and NEM were the reagents, free cysteine was omitted, owing to re-activation of the enzyme with 10 mM-cysteine. After stopping the reaction, dialysis was carried out at 4 °C overnight and the enzyme was then assayed.

Table 3. Effect of various modifying reagents on intestine and kidney maltase–glucoamylase

Incubation times and conditions were as stated in Table 2. Abbreviations: M, maltase; G, glucoamylase; na, no activity.

Activity of maltase-glucosylase at different concentrations (mM) of modifying reagents (%) :																	
	Modifying reagent	Control		0.001		0.002		0.005		0.01		0.025		0.2		0.25	
		M	G	M	G	M	G	M	G	M	G	M	G	M	G	M	G
Intestine	DEP	100	100														
	NBS	100	100	61±1.7	80±1.0	30±2.7	53±1.0	29±1.0	19±2.0	14±1.2	19±1.5	na	19±1.0			57±1.7	70±1.5
	HNBB	100	100														
	TNBS	100	100														
	Pyridoxal phosphate	100	100														
Kidney	DEP	100	100														
	NBS	100	100	76±1.0	75±1.0	75±1.7	77±2.0	75±2.7	80±1.5	29±1.0	27±1.2	5±1.0	8±1.0			63±1.2	68±1.0
	HNBB	100	100														
	NEM	100	100														
	PHMB	100	100											37±1.5	33±2.1		
	Modifying reagent	Control		0.5		1.0		2.0		4.0		5.0		10.0		20.0	
		M	G	M	G	M	G	M	G	M	G	M	G	M	G	M	G
Intestine	DEP	100	100	56±2.7	44±2.0	50±1.2	15±1.0	13±1.2	na			na	na	na	na		
	NBS	100	100														
	HNBB	100	100			36±2.0	20±1.0	na	na	na	na	na	na	na	na	na	na
	TNBS	100	100			63±3.0	44±2.0					44±1.0	38±1.7				
	Pyridoxal phosphate	100	100	70±2.1	74±1.7	49±1.0	28±1.7	19±1.2	11±1.0	na	na						
Kidney	DEP	100	100	55±2.7	57±1.7	43±1.2	52±1.0	na	na			na	na	na	na		
	NBS	100	100														
	HNBB	100	100			84±3.0	82±1.0	57±2.7	52±1.5	42±2.0	na	42±1.2	na				
	NEM	100	100			77±1.0	72±2.0					70±1.7	62±1.0	62±2.0	43±1.5		
	PHMB	100	100	36±1.7	32±2.7	20±2.0	21±1.0	20±1.0	19±1.2								

For pseudo-first-order rate kinetics, residual activity at different time intervals was determined.

Protection by substrates

Protection studies were done by preincubating the enzyme with different concentrations of substrates maltose and starch at 37 °C for 5 min, followed by addition of the protein-modifying reagent. The enzyme activity was assayed after dialysis.

Reversal with hydroxylamine

The DEP- and TNBS-modified inactivated enzymes were treated with 100 mM-hydroxylamine (pH 7.0) at 37 °C for 4 h,

dialysed at 4 °C overnight and assayed (Burstein *et al.*, 1974; Riordan & Vallee, 1972).

Reversal with cysteine

The PHMB- and NEM-modified inactivated enzyme was treated with 10 mM-cysteine at 37 °C for 30 min, dialysed at 4 °C overnight and assayed.

Spectral studies

Absorption spectra were recorded on a Shimadzu UV-160 spectrophotometer. All enzyme solutions were previously dialysed extensively against the appropriate buffers.

Fig. 1. Pseudo-first-order plots for the inactivation by NBS of (a) maltase and (b) glucoamylase from intestine and (c) maltase and (d) glucoamylase from kidney

Concentrations of NBS were 0.001 mM (●), 0.002 mM (■), 0.005 mM (○) and 0.01 mM (▲). v is the enzyme activity remaining at any given time, and v_0 is the activity of the uninhibited enzyme. Insets: second-order plots of the pseudo-first-order rate constants (K_{app}) (min^{-1}) of inactivation at different concentrations (mM) of NBS.

RESULTS AND DISCUSSION

Complete inhibition by DEP, NBS, HNBB, PHMB and NEM indicated that histidine, tryptophan and cysteine residues may be essential for the activity of the kidney enzyme. Inhibition by DEP, NBS, HNBB, TNBS and pyridoxal phosphate indicated that histidine, tryptophan and lysine residues may be involved in the active site of the intestinal enzyme. No significant change in activity was observed with different concentrations of the other modifying reagents (Table 3).

Kinetic studies had indicated the possible presence of histidine and cysteine at the active site of the renal enzyme (Pereira & Sivakami, 1989). The present study, besides confirming these observations, also indicated a third residue, tryptophan, at the active site. The presence of histidine and tryptophan at the active site seems to be common to the intestine and kidney enzymes.

Plots of log of residual activity as a function of time at various concentrations of modifiers indicated that the inactivation process exhibited pseudo-first-order kinetics with respect to time at a fixed concentration of the inhibitor (Fig. 1). Applying the analysis described by Levy *et al.* (1963) and multilinear regression analysis, the pseudo-first-order rate constants (K_{app}) were calculated from the slope of the plots of log of residual activity [$\log(v/v_0)$] against the time of the reaction. The order of the reaction was estimated from the slope of the plots of log(pseudo-first-order rate constant, K_{app}) against log(inhibitor concentration). These graphs (Fig. 1 insets) indicated that inactivation of the maltose-hydrolytic activity resulted from the reaction of one histidine (or one molecule of DEP), one tryptophan (or one molecule of NBS/HNBB) and one cysteine (or one molecule of PHMB/NEM) residue per molecule of enzyme, whereas starch-hydrolytic activity appeared to require in addition to these another histidine residue (or an additional molecule of DEP for inactivation). With the intestinal enzyme, inactivation of maltose-hydrolytic activity resulted from reaction of one molecule of DEP, one molecule of NBS/HNBB and one molecule of TNBS/pyridoxal phosphate per molecule of enzyme, and probably two molecules of DEP, one molecule of NBS/HNBB and one molecule of TNBS/pyridoxal phosphate per molecule of glucoamylase (Table 4). Probably the second histidine residue specific to the hydrolysis of starch is involved in binding and alignment of the larger starch chain, or possibly the maltose-binding site is a subsite within the larger catalytic site for starch, as seen in the kinetic studies on the intestinal enzyme (Sivakami & Radha-

krishnan, 1976). From these data, a second-order rate constant (k) for the modifiers was calculated (Levy *et al.*, 1963) (Table 4). From these second-order rate constants it was observed that the rate of inactivation of glucoamylase was higher than that for maltase. The starch-binding site appears to be more labile than the maltose-binding site. Moreover, the intestinal enzyme appears to undergo inactivation at a higher rate than the renal enzyme (Table 4). More than half-maximal protection, from 75 % to the extent of 100 %, was given by maltose at concentrations of 1 mM (nearly equal to the K_m of 0.67 mM for maltose), protection increasing with concentrations of 5 mM and 10 mM, and by starch at concentrations of 0.2, 0.5 and 1.0 mg/ml, values less than the K_m for starch (1.5 mg/ml), suggesting the involvement of histidine, tryptophan and lysine residues at the substrate-binding site in the intestinal enzyme, and histidine, tryptophan and cysteine in the renal enzyme. The 100 % protection by starch at concentrations less than the K_m may be attributed to the heterogeneity of starch, which results in an 'apparent' value for the K_m , which is also seen for other carbohydrases, such as xylanase protection by xylan (Keskar *et al.*, 1989) (Table 5). With increasing concentrations of substrate, 100 % protection was observed against most of the modifying reagents, except for DEP, where maltose protected nearly totally, glucoamylase from kidney as well as intestine was protected by starch to the extent of 75–80 % (Table 5).

Moreover, although reversal with hydroxylamine was 100 % for maltase activity, it was only 70 % for glucoamylase activity. This suggests that one histidine residue is probably present in the maltose-binding subsite, but the other is outside this subsite, but within the larger catalytic site for starch. Hydroxylamine did not restore the activity of the TNBS-modified intestinal enzyme, confirming the presence of lysine. Complete re-activation of the PHMB/NEM-modified renal enzyme by cysteine was observed, indicating a competitive displacement of PHMB and NEM by high concentrations of thiol (Means & Feeney, 1971). Tryptophan has been shown to be present in the active site of several other carbohydrases, such as xylanase (Keskar *et al.*, 1989), lysozyme and cellulase (Imoto *et al.*, 1972; Yaguchi *et al.*, 1983), owing to its ability to form hydrogen bonds with sugars.

A characteristic absorption peak at 242 nm in the protein difference spectrum for the DEP-modified intestinal and renal enzymes was observed. A minimum in the region of 280 nm was not observed (Miles, 1977). A sharp decrease in A_{280} for the NBS-modified intestinal and renal enzymes (Spande & Witkop,

Table 4. Second-order rate constants (k) for the intestinal and kidney maltase–glucoamylase with the various reagents

Number of molecules of modifier reacting per enzyme molecule (n) is calculated by multilinear regression analysis. Abbreviations: M, maltase; G, glucoamylase.

	Intestine				Kidney			
	n		k (mm ⁻¹ ·min ⁻¹)		n		k (mm ⁻¹ ·min ⁻¹)	
	M	G	M	G	M	G	M	G
DEP	0.7±0.05	1.4±0.09	0.044±0.002	0.07±0.002	0.98±0.03	1.57±0.02	0.018±0.002	0.021±0.001
NBS	1.0±0.1	1.0±0.1	12.0±1.2	13.0±1.6	0.6±0.02	0.8±0.014	1.6±0.12	2.4±0.1
HNBB	0.9±0.1	1.0±0.1	0.11±0.007	0.11±0.01	0.6±0.02	1.1±0.07	0.034±0.002	0.06±0.005
NEM	—	—	—	—	0.5±0.03	0.5±0.007	0.016±0.001	0.011±0.001
PHMB	—	—	—	—	1.1±0.05	0.7±0.03	0.075±0.001	0.063±0.002
TNBS	0.5±0.03	0.5±0.007	0.014±0.001	0.011±0.001	—	—	—	—
Pyridoxal phosphate	0.8±0.03	1.26±0.04	0.03±0.002	0.035±0.002	—	—	—	—

Table 5. Effect of substrates on the inactivation of maltase-glucosylase by protein-modifying reagents

Abbreviations: M, maltase; G, glucosylase.

Intestinal maltase-glucoamylase activity (%) in the presence of:				Kidney maltase-glucoamylase activity (%) in the presence of:												
Modifying reagent	Concn. (mM)	Enzyme	Modifying reagent	Maltose (mM)			Starch (mg/ml)			Modifying reagent	Maltose (mM)			Starch (mg/ml)		
				1	5	10	0.2	0.5	1.0		1	5	10	0.2	0.5	1.0
HNBB	5.0	M	0 ± 0	90 ± 1.2	100 ± 1.0	100 ± 0.7	77 ± 1.0	100 ± 0.7	100 ± 0.7	33 ± 1.6	80 ± 1.2	88 ± 1.5	100 ± 0.7	87 ± 1.2	100 ± 1.2	100 ± 1.5
		G	0 ± 0	100 ± 0.7	100 ± 1.0	100 ± 0.0	100 ± 2.0	100 ± 0.7	100 ± 1.0	0 ± 0.0	90 ± 1.7	100 ± 1.0	100 ± 1.0	100 ± 1.0	100 ± 1.2	100 ± 1.2
NBS	0.025	M	10 ± 0.7	91 ± 1.2	99 ± 1.5	100 ± 0.7	100 ± 2.1	100 ± 1.2	100 ± 1.0	0 ± 0.0	81 ± 0.7	93 ± 1.2	96 ± 1.2	86 ± 1.0	95 ± 1.0	100 ± 1.5
		G	11 ± 0.5	78 ± 1.2	77 ± 1.0	100 ± 0.7	100 ± 1.0	100 ± 0.7	100 ± 0.0	0 ± 0.0	84 ± 1.7	85 ± 1.5	88 ± 0.7	78 ± 1.5	100 ± 0.7	100 ± 0.5
DEP	5.0	M	0 ± 0	98 ± 1.0	100 ± 1.0	100 ± 1.0	79 ± 2.1	79 ± 1.0	80 ± 0.7	0 ± 0.0	90 ± 1.0	90 ± 1.2	100 ± 1.0	67 ± 1.2	79 ± 1.2	88 ± 0.7
		G	0 ± 0	95 ± 2.0	100 ± 0.7	100 ± 0.7	76 ± 1.2	79 ± 1.2	79 ± 1.0	0 ± 0.0	85 ± 0.7	86 ± 1.0	100 ± 1.5	75 ± 0.7	86 ± 1.2	88 ± 1.0
PHMB	1.0	M	-	-	-	-	-	-	-	26 ± 0.7	80 ± 2.0	86 ± 1.2	100 ± 1.5	95 ± 1.0	100 ± 2.0	100 ± 1.0
		G	-	-	-	-	-	-	-	12 ± 1.0	80 ± 1.5	98 ± 0.7	100 ± 0.7	96 ± 1.0	100 ± 1.2	100 ± 0.7
NEM	10.0	M	-	-	-	-	-	-	-	58 ± 1.2	90 ± 1.0	90 ± 1.2	98 ± 1.0	89 ± 2.0	100 ± 2.1	100 ± 1.0
		G	-	-	-	-	-	-	-	56 ± 2.0	86 ± 0.7	100 ± 1.0	100 ± 1.0	100 ± 1.2	100 ± 1.5	100 ± 1.0
TNBS	20.0	M	0 ± 0	92 ± 2.1	92 ± 0.7	100 ± 1.0	83 ± 2.1	93 ± 2.0	100 ± 1.2	-	-	-	-	-	-	-
		G	0 ± 0	78 ± 0.7	78 ± 1.7	80 ± 1.2	72 ± 0.7	80 ± 0.7	96 ± 1.0	-	-	-	-	-	-	-
Pyridoxal phosphate	4.0	M	0 ± 0	88 ± 2.0	90 ± 1.5	100 ± 1.0	100 ± 2.0	100 ± 1.2	100 ± 0.5	-	-	-	-	-	-	-
		G	0 ± 0	80 ± 1.7	80 ± 1.0	84 ± 0.7	84 ± 1.5	87 ± 1.0	100 ± 1.2	-	-	-	-	-	-	-

1967), and an increase in A_{410} for the HNBB-modified enzymes, was observed (Horton & Koshland, 1972). The NEM A_{305} maximum was abolished for the modified renal enzyme, and an increase in A_{250} upon formation of the mercaptide with PHMB was observed (Riordan & Vallee, 1972). A shift in absorbance from the 388 nm maximum for pyridoxal phosphate to 412 nm for the modified intestinal enzyme was observed (Means & Feeney, 1971).

This is the first report of a systematic study of the active site of a brush-border glycosidase.

B.P. thanks the University Grants Commission (UGC), New Delhi, for the award of a Senior Research Fellowship. This work was carried out under a UGC Scheme awarded to S.S.

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