BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **238,** 896–898 (1997)
ARTICLE NO. RC977148

Human Lysosomal and Jack Bean α-Mannosidases Are Retaining Glycosidases

Steven Howard,* Curtis Braun,* John McCarter,* Kelley W. Moremen,† Yung-Feng Liao,† and Stephen G. Withers*,[1]

**Protein Engineering Network of Centres of Excellence of Canada and the Department of Chemistry, University of British Columbia, Vancouver, B.C. Canada, V6T 1Y6; and †Complex Carbohydrate Research Center and Department of Biochemistry and Molecular Biology, University of Georgia, Athens, Georgia 30602*

Received July 7, 1997

The stereochemical course of the hydrolyses catalysed by two α-mannosidases has been determined directly by ^{1}H NMR. Synthetic substrates were incubated with the enzymes and the anomeric configuration of the initially formed product was ascertained in each case by observation of the chemical shift of the anomeric proton at the hemiacetal centre. Both mannosidases were found to catalyse hydrolysis with retention of stereochemistry at the anomeric position. Human lysosomal α-mannosidase (a class II mannosidase) is a member of the glycosidase family 38 and thus has sequence similarity with several α-mannosidases responsible for glycoprotein biosynthesis. Jack bean α-mannosidase was shown to be mechanistically similar to the lysosomal enzyme and will provide a useful model system in mechanistic studies and inhibitor design.

Human lysosomal α-mannosidase is responsible for the catabolism of N-glycans on glycoproteins (1). It has a broad specificity which includes the hydrolysis of α(1,2), α(1,3) and α(1,6) mannoside linkages present in complex, hybrid and high mannose Asn-linked glycans (2, 3). A deficiency in this enzyme gives rise to the genetic disease, α-mannosidosis, which results in a proliferation of lysosomes caused by the accumulation of undegraded oligosaccharides (3, 4, 5). Recently it has been shown that lysosomal α-mannosidase shows regions of sequence similarity with other mammalian α-mannosidases such as Golgi α-mannosidases and the endoplasmic reticulum/cytosolic α-mannosidases and these enzymes have been categorised as class II mannosidases or family 38 glycosidases in Henrissat's classification (6, 7). There has been widespread interest in these enzymes, particularly the Golgi α-mannosidase, as inhibitors of this enzyme show anticancer activity (8). However, no sequence similarity was found with the endoplasmic reticulum and Golgi α(1,2)-mannosidases, involved in early steps of glycoprotein biosynthesis, which have been categorised as class I mannosidases or glycosidase family 47 (6, 7, 9).

Although human lysosomal α-mannosidase has been expressed in *Pichia pastoris,* attempts to express other enzymes such as the rat Golgi α-mannosidase at useful levels have not been successful (6, 10). At present, samples of these enzymes are extremely valuable and in many cases large quantities are not available, thus making mechanistic and structural studies difficult. Fortunately, jack bean α-mannosidase is commercially available and may provide a convenient model system for the more valuable mammalian enzymes. Jack bean α-mannosidase catalyses hydrolysis of α(1,2)-, α(1,3)- and α(1,6)-linkages between mannose residues but also has a broad aglycone specificity, including aryl groups (11).

A knowledge of the stereochemical outcome of substrate hydrolysis is an essential prerequisite for mechanistic studies on these enzymes. α(1,2)-Mannosidase (class I or family 47) from *Saccharomyces cerevisiae* has been shown to be an inverting enzyme, i.e. the initially formed product is β-D-mannose, which has the opposite anomeric configuration compared to the substrate (14). Since other studies (12, 13) have established that all members of a sequence related family hydrolyse their substrates with the same stereochemical outcome, this result established family 47 as an inverting family. No such experiment has been done with the class II or the well-studied jack bean α-mannosidases. This paper describes the use of ^{1}H NMR spectrometry to directly observe the stereochemical courses of these enzymatic reactions.

[1] To whom correspondence should be addressed. Fax: (604) 822-2847. E-mail: withers@chem.ubc.ca.

Abbreviations: NMR, nuclear magnetic resonance; PNPM, *p*-nitrophenyl-α-D-mannopyranoside; DNPM, 2,4-dinitrophenyl-α-D-mannopyranoside.

0006-291X/97 $25.00

MATERIALS AND METHODS

^{1}H NMR spectrometry was performed on a Bruker WH-400 at 400 MHz using 5 mm tubes. Experiments were conducted at ambient temperature using DSS as external standard.

All buffer chemicals, PNPM and jack bean α-mannosidase were obtained from Sigma Chemical Company. DNPM was synthesised in one step by treatment of D-mannose with 1-fluoro-2,4-dinitrobenzene (15). Human lysosomal α-mannosidase was expressed in *Pichia pastoris* and purified as previously reported (10).

PNPM (1.7 mg) was lyophilized twice from 99.9% D_2O and twice from 99.96% D_2O. Sodium citrate buffer (50 mM, pH 4.5) was lyophilized in the same way and was finally dissolved in an equal volume of 99.996% D_2O. Jack bean α-mannosidase (5 mg, 100 U) was dialysed against 99.9% deuterated buffer (50 mM citrate, pH 6.8) (400 μl) using a centrifugal ultrafilter with a molecular weight cut-off of 30,000 (Millipore) by concentration to 50 μl, then dilution with fresh buffer to 400 μl (16). This was repeated three more times. The enzyme was then washed twice more with 99.96% (pH 4.5) deuterated buffer, diluted to 500 μl with 99.996% deuterated buffer (pH 4.5) and reduced to 50 μl by ultrafiltration. The PNPM was dissolved in 0.7 mL 99.996% deuterated buffer (pH 4.5), to give a concentration of 8.1 mM, and added to an NMR tube (dried in dessicator). After initial acquisition of the PNPM spectrum, the enzyme was added and a spectrum acquired approximately every 4 min.

Human lysosomal α-mannosidase (0.16 mg, 0.4 U) was incubated with DNPM (27 mM) in sodium citrate buffer (50 mM, pH 4.5). The deuterated solutions were prepared in an analogous fashion to that used for jack bean α-mannosidase.

RESULTS AND DISCUSSION

^{1}H NMR spectrometry was used to determine the stereochemistry of the initially formed products from the enzymatic hydrolysis of synthetic substrates. Jack bean α-mannosidase was incubated with 8.1 mM PNPM and the spectrum acquired at $\approx$ 4 min. intervals as shown in Figure 1. Although jack bean α-mannosidase requires zinc for full activity and stability (16), zinc sulfate was omitted from the reaction since substantial amounts of Zn^{2+} were found to catalyze the mutarotation of the initially released mannose product and cause rapid equilibration between α and β-mannose. Figure 1a shows an expansion of the PNPM spectrum prior to the addition of enzyme where the singlet at δ 5.75 ppm corresponds to the anomeric proton and the large signal at $\approx \delta$ 4.7 ppm is a result of residual DOH. Figure 1b shows the spectrum 3 min. after the addition of jack bean α-mannosidase, at which time the reaction was almost complete. The anomeric proton of α-mannose is clearly present at δ 5.15 ppm, whereas no signal in the δ 4.85-4.90 region (β-mannose) was evident. At 23 min. (Figure 1c), the reaction was complete and the product was predominantly α-mannose with a small amount of β-mannose, δ 4.86 ppm, which had formed due to mutarotation of α-mannose. After 96 min, the spectrum indicated that mutarotation had given a fully equilibrated mixture of α- and β-mannose (Figure 1d).

In an analogous experiment, human lysosomal α-mannosidase was incubated with 27 mM DNPM and the reaction monitored by ^{1}H NMR spectrometry. The initially formed product was also found to be α-mannose.

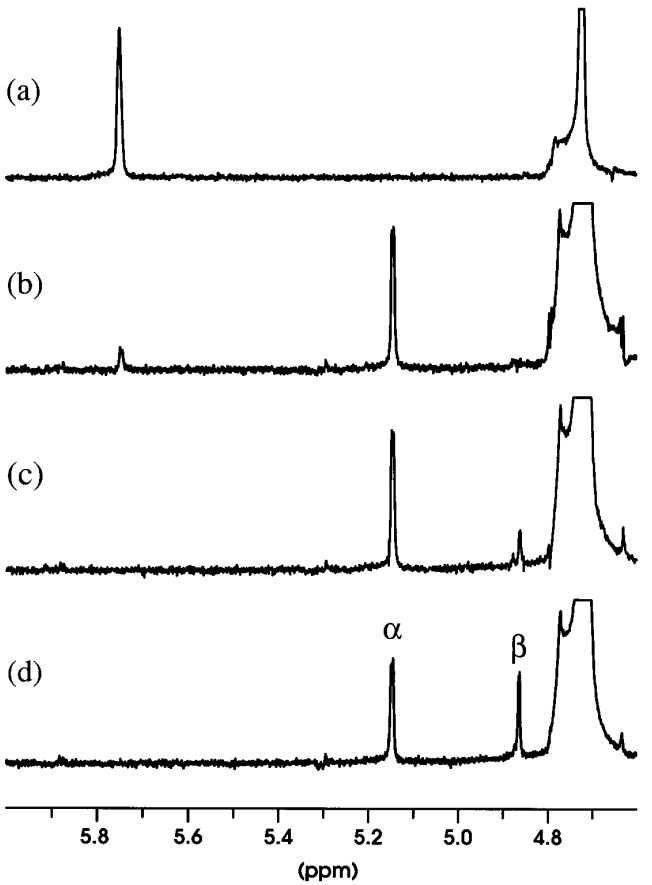

FIG. 1. Partial ^{1}H NMR spectra of a reaction mix containing PNPM and jack bean α-mannosidase at different times of reaction. (a) Before addition of enzyme; (b) 3 min after enzyme addition; (c) 23 min after; and (e) 96 min after enzyme addition.

It is clear that these two mannosidases are retaining enzymes and are therefore assumed to employ a double-displacement mechanism. Since lysosomal α-mannosidase shows large regions of sequence similarity with other class II mammalian α-mannosidases, it seems reasonable to expect that all the class II enzymes, thus all enzymes of family 38, are retaining. Further, since only two families of α-mannosidases have been identified to date, families 38 and 47, of which the former are retaining as exemplified by the human lysosomal enzyme and the latter are inverting (14), it is quite possible that the jack bean enzyme, being a retaining enzyme, may well also be a member of family 38. However, sequence information will be required to confirm this prediction.

Whatever the structure, the discovery that the lysosomal α-mannosidase, thus likely also the Golgi mannosidase II, are retaining enzymes provides mechanistic insight which should prove useful in the design of

specific inhibitors which might be of therapeutic value. One approach which could prove useful could be the use of 5-fluoro-α-D-mannosyl fluoride to at least transiently trap the glycosyl-enzyme intermediate formed on the retaining glycosidase, as has been shown with yeast α-glucosidase (17, 18). This could not only provide good inhibitors, but also might provide a means for identifying the catalytic nucleophile at the active site.

Jack bean α-mannosidase, being a retaining mannosidase, should prove a useful model system to test new labeling techniques since this enzyme is more readily available than the mammalian α-mannosidases and is easily assayed with PNPM or DNPM.

ACKNOWLEDGMENTS

We thank the Protein Engineering Network of Centres of Excellence of Canada and GlycoDesign Inc. for financial support of this work. We are also grateful for NIH Research Grants GM 47533 and RO 5351 to K.W.M.

REFERENCES

1. Aronson, N. N., Jr., and Kuranda, M. J. (1989) *FASEB J.* **3,** 2615–2622.
2. al Daher, S., de Gasperi, R., Daniel, P., Hall, N., Warren, C. D., and Winchester, B. (1991) *Biochem. J.* **277,** 734–751.
3. Daniel, P. F., Winchester, B., and Warren, C. D. (1994) *Glycobiology* **4,** 551–566.
4. Bennet, J. K., Dembure, P. P., and Elsas, L. J. (1995) *Am. J. Med. Genet.* **55,** 21–26.
5. Neufeld, E. F. (1991) *Annu. Rev. Biochem.* **60,** 257–280.
6. Moremen, K. W., Trimble, R., and Herscovics, A. (1994) *Glycobiology* **4,** 113–125.
7. Henrissat, B., and Bairoch, A. (1996) *Biochem. J.* **316,** 695–696.
8. Gross, P. E., Baker, M. A., Carver, J. P., and Dennis, J. W. (1995) *Clinical Cancer Res.* **1,** 935–944.
9. Lipari, F., and Herscovics, A. (1994) *Glycobiology* **4,** 697–702.
10. Liao, Y-F., Lal, A., and Moremen, K. W. (1996) *J. Biol. Chem.* **271,** 28348–28358.
11. Li, Y-T. (1967) *J. Biol. Chem.* **242,** 5474–5480.
12. Davies, G., and Henrissat, B. (1995) *Structure* **3,** 853–859.
13. Gebler, J., Gilkes, N. R., Claeysssens, M., Wilson, D. B., Beguin, P., Wakarchuk, W. W., Kilburn, D. G., Miller, Jr., R. C., Warren, A. J., and Withers, S. G. (1992) *J. Biol. Chem.* **267,** 12559–12561.
14. Lipari, F., Gour-Salin, B. J., and Herscovics, A. (1995) *Biochem. Biophys. Res. Commun.* **209,** 322–326.
15. Sharma, S. K., Corrales, G., and Penades, S. (1995) *Tetrahedron Lett.* **36,** 5627–5630.
16. Snaith, S. M. (1975) *Biochem. J.* **147,** 83–90 [Sodium citrate buffer (pH 6.8) was used for the initial deuterium exchanges since the enzyme is unstable at lower pH in the absence of Zn^{2+}.]
17. McCarter, J., and Withers, S. G. (1996) *J. Am. Chem. Soc.* **118,** 241–242.
18. McCarter, J., and Withers, S. G. (1996) *J. Biol. Chem.* **271,** 6889–6894.