Mechanism-based Inhibition of Yeast $\alpha$-Glucosidase and Human Pancreatic $\alpha$-Amylase by a New Class of Inhibitors

2-DEOXY-2,2-DIFLUORO-$\alpha$-GLYCOSIDES*

(Received for publication, August 28, 1995)

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2-Deoxy-2,2-difluoroglycosides are a new class of mechanism-based inhibitors of $\alpha$-glycosidases, which function via the accumulation of a stable difluoroglycosyl-enzyme intermediate. Two members of this new class of inhibitor have been synthesized and kinetic studies performed with their target glycosidases. Thus 2,4,6-trinitrophenyl 2-deoxy-2,2-difluoro-$\alpha$-glucoside is shown to inactivate yeast $\alpha$-glucosidase with a second order rate constant of $k_i/K_i = 0.25 \text{ min}^{-1} \text{ mm}^{-1}$. The equivalent difluoromaltoside inactivates human pancreatic $\alpha$-amylase with $k_i/K_i = 0.0073 \text{ min}^{-1} \text{ mm}^{-1}$. Competitive inhibitors protect the enzyme against inactivation in each case, showing reaction to occur at the active site. A burst of release of one equivalent of trinitrophenolate occurs, showing reaction to occur at the active site, a burst of release of one equivalent of trinitrophenolate occurs, showing reaction to occur at the active site. A burst of release of one equivalent of trinitrophenolate occurs, showing reaction to occur at the active site.

Specific inhibitors of glycosidases have proved valuable in a number of applications ranging from mechanistic studies (Legler, 1990; Sinnott, 1990) through their use to study protein glycosylation (Elbein et al., 1984), to possible therapeutic uses such as the control of blood glucose levels via control of the degradation of dietary disaccharides and starch (Truscott et al., 1981) or control of viral infectivity through interference with normal glycosylation of viral coat proteins (Elbein, 1984; Prasad et al., 1987). A number of naturally occurring reversible glycosidase inhibitors are known such as noirimycin, castanospermine, swainsonine, and acarbose (Legler, 1990), and these have been subjected to intensive study including the synthesis and testing of a number of analogues. Another class of inhibitors that has been less well studied is that of the covalent, irreversible type, typically affinity labels. These are generally synthetic analogues of sugars containing reactive groups such as epoxides, isothiocyanates and $\alpha$-halocarbonyls as reviewed recently (Legler, 1990; Withers and Aebersold, 1995). Less common are the more selective mechanism-based inhibitors whose efficacy depends upon binding and subsequent enzymatic action to generate a reactive species. These include the conduritol epoxides (Legler, 1968, 1970), the guanine methide-generating glycosides (Halazy et al., 1990; Briggs et al., 1992), and the glycosylmethyl triazenes (Marshall et al., 1980; Sinnott and Smith, 1976). Interestingly, two naturally occurring inhibitors of this class have now been described: the hydroxymethylconduritol epoxide, cyclophellitol (Atsumi et al., 1990; Withers and Umezawa, 1991), isolated from Phellinus sp.; and the putative quinone methide-generating glycoside salicortin, isolated from Salix (Clausen et al., 1990).

An additional, recently described class of mechanism-based inhibitor that has proved successful is that of the 2-deoxy-2-fluoro (Withers and Aebersold, 1995; Withers et al., 1987, 1988, 1990). These function as excellent inactivators of retaining glycosidases (glycosidases that hydrolyze the glycosidic linkage with net retention of anomeric configuration) by formation of a stable glycosyl-enzyme intermediate which turns over to product only very slowly. As shown in Scheme 1 for an $\alpha$-glucosidase, the normal mechanism of action of this class of enzyme involves the formation and hydrolysis of a glycosyl-enzyme intermediate with general acid/base catalytic assistance via transition states with substantial oxocarbenium ion character (Sinnott, 1987, 1990). A combination of inductive destabilization of these positively charged transition states by the electronegative fluoride at C-2 and loss of crucial hydrogen bonding interactions with the 2-hydroxyl serves to substantially destabilize these two transition states, dramatically slowing both steps. Incorporation of a relatively reactive leaving group such as fluoride or 2,4-dinitrophenolate as aglycone accelerates the first (glycosylation) step sufficiently that the glycosyl-enzyme intermediate is formed, but then hydrolyzes only very slowly, thereby resulting in inactivation.

This strategy has proved very successful with a wide range of retaining $\beta$-glycosidases, and has allowed the characterization of this intermediate and the identification of the catalytic nucleophiles in a number of enzymes (Withers and Aebersold, 1995). These compounds have also proved effective in vivo, selectively inactivating the expected glycosidases in all organs tested in rats, including the brain (McCartter et al., 1994). However, as noted early on (Withers et al., 1988), and as has been confirmed in subsequent studies (McCartter et al., 1993), this approach has not been successful with $\alpha$-glycosidases, despite the fact that ample evidence exists that equivalent mechanisms are followed in the two cases.1 This evidence includes the $^{13}$C NMR detection of a glycosyl-enzyme intermediate on an $\alpha$-amylase (Tao et al., 1989), and the denaturation trapping of such an intermediate on a glycosyl transferase, a mechanistically analogous enzyme (Mooser et al., 1991; Mooser, 1992). Instead, the 2-deoxy-2-fluoro-$\alpha$-glycosides function as substrates, albeit poor, for the enzymes studied. Thus the lack of inactivation must be a consequence of the fluorine substituent not sufficiently slowing the glycosylation step relative to hydrolysis, and a possible stereoelectronic rationale for this.

1 Very slow inactivation was seen in some cases. This has subsequently been shown to be due to a low level ($<0.1\%$) contaminant of a highly effective inactivator in the 2-fluoroglycoside sample.
2,2-Difluoroglycosides as α-Glycosidase Inhibitors

2,2-Difluoroglycosides have been developed as α-glycosidase inhibitors. The generation of an effective inhibitor of this class for an α-glycosidase therefore requires further slowing of the deglycosylation step. This could be achieved via the incorporation of a second fluorine at C-2 to further inductively destabilize the transition state, in conjunction with the incorporation of a more reactive leaving group to ensure that glycosylation is not rate-limiting. Since the second fluorine is only slightly larger than the hydrogen it replaces, it would likely not result in any significant steric repulsive interactions upon binding. In this paper we describe such an approach which has led to the development of novel mechanism-based inactivators of both yeast α-glucosidase and human pancreatic α-amylase.

This is the first mechanism-based inhibitor described for human pancreatic α-amylase, which should prove valuable as a probe of the structure and mechanism of this medically important enzyme. Compounds of this general class also have considerable potential as therapeutics agents, particularly in the control of post-prandial blood glucose levels by inhibition of digestive glycosidases.

MATERIALS AND METHODS

Synthesis—Syntheses of the two inhibitors will be described in detail elsewhere. Characterization data for the final compounds are provided below.

2,4,6-Trinitrophenyl 2-deoxy-2,2-difluoro-α-D-arabino-hexopyranoside (TNPDFG)2 data were as follows: 1H NMR (D2O): δ 9.14 (s, 2 H, aryl Hs), 5.89 (d, 1 H, J 3,4 = 19.6 Hz, H-1), 4.16 (dt, 1 H, J 3,4 = 7.5 Hz); 19F NMR (D2O): δ -120.6 (dd, J F2a,F2e = 6 Hz, J Fa-2). 1.5 H2O

Calculated: C 34.67 H 2.79 N 10.10

Found: C 34.68 H 2.91 N 9.89

2,4,6-Trinitrophenyl 2-deoxy-2,2-difluoro-4-O-(α-(1, 4)-α-glucosyl)-α-

2 The abbreviations used are: TNPDFG, 2,4,6-trinitrophenyl 2-deoxy-2,2-difluoro-α-D-glucoside; TNPDFM, 2,4,6-trinitrophenyl 2-deoxy-2,2-difluoro-α-D-maltoside; TNPDFA, 2,4,6-trinitrophenyl 2-deoxy-2,2-difluoro-α-D-arabino-hexopyranoside.

RESULTS AND DISCUSSION

Synthesis of the required glycone portion posed no great synthetic problem since a method for the synthesis of a 2-deoxy-2,2-difluoroglucose derivative based upon the addition of a leaving group to 2-fluoro-2-D-glucal had been published (McCarter et al., 1993). However, the incorporation of an aglycone of greater leaving group ability than fluoride or dinitrophenolate necessitated more careful synthetic considerations. The most attractive candidate as a leaving group was chloride. However, repeated attempts to synthesize this derivative via displacement chemistry were unsuccessful, with no reaction occurring. This is likely the consequence of the very effect sought, the resistance of 2,2-difluoroglycosides toward nucleophilic displace-
ments. An alternative strategy for the installation of a good leaving group was therefore followed, which did not require the displacement reaction. This involved reaction of the protected hemiacetal of the 2-deoxy-2,2-difluoro sugar with fluoro-2,4,6-trinitrobenzene, to yield the trinitrophenyl glycoside. Using this approach TNPDFG (1) and TNPDFM (2) were synthesized as shown in Structure 1.

Treatment of 3,4,6-tri-O-acetyl 2-deoxy-2-fluoro-α-D-glucopyranosyl bromide (McCarter et al., 1993) with triethylamine in acetonitrile at reflux for 20 h yielded 2-fluoro-α-D-glucal, which was fluorinated with acetyl hypofluorite to give 1,3,4,6-tetra-O-acetyl 2-deoxy-2,2-difluoro-α-D-glucopyranose. Selective anomic deprotection with hydrazine acetate in dimethyl formamide at 50°C yielded the free hemiacetal after 3 days. This was reacted with fluoro-2,4,6-trinitrobenzene in dichloromethane at 50°C yielding the free hemiacetal after 3 days. This involved reaction of the protected leaving group was therefore followed, which did not require the displacement reaction. This involved reaction of the protected hemiacetal of the 2-deoxy-2,2-difluoro sugar with fluoro-2,4,6-trinitrobenzene, to yield the trinitrophenyl glycoside. Using this approach TNPDFG (1) and TNPDFM (2) were synthesized as shown in Structure 1.

Inactivation data were analyzed according to the kinetic scheme shown below (Reaction 1).

\[ E + TNPDFM \rightarrow E \cdot TNPDFM \]
\[ E \cdot TNPDFM \rightarrow E \cdot DFM \rightarrow E + DFM \]

1. **Species E, TNP, and DFM represent, respectively, enzyme, 2,4,6-trinitrophenol and 2-deoxy-2,2-difluoro-α-maltose or 2-deoxy-2,2-difluoro-α-glucose. A reciprocal replot of the pseudo-first order rate constants \( k_{p} \) at each inhibitor concentration, taken from slopes of the lines in Fig. 1a versus inhibitor concentration, is shown in Fig. 1b. The slope of this plot yielded a value of \( k_{p} / K_{i} = 0.0073 \ \text{min}^{-1} \ \text{mM}^{-1} \) for reaction of human pancreatic α-amylase with TNPDFM.

Inactivation data were analyzed according to the kinetic scheme shown below (Reaction 1).
2,2-Difluoroglycosides as α-Glycosidase Inhibitors

Kinetic parameters for human pancreatic α-amylase and yeast α-glucosidase

| Enzyme       | Substrate/inhibitor | $K_m$ or $K_i$ | $k_{cat}$ or $k_i$ | $k_{cat}/K_m$ or $k_i/K_i$ |
|--------------|---------------------|--------------|----------------|----------------------------|
| α-Amylease   | MF                  | 4.5 ± 0.1    | 26.580 ± 300     | 5.880 ± 180                |
| α-Amylease   | FFM                 | 4.7 ± 0.9    | 10.2 ± 1.2       | 2.4 ± 0.6                  |
| α-Amylease   | TNPDFM              | NA          | NA              | 0.0073                     |
| α-Glucosidase| GF                  | 0.93         | 1,468           | 1,600                      |
| α-Glucosidase| FFM                 | 4.8 ± 0.8    | 96 ± 6          | 20 ± 5                     |
| α-Glucosidase| TNPDFM              | NA          | NA              | 0.25                       |

* MF, α-mannosyl fluoride; FFM, 2-fluoro-α-mannosyl fluoride; GF, α-glucosyl fluoride; FGF, 2-fluoro-α-glucosyl fluoride.

* NA, not available.

* Taken from Konstantinidis et al. (1991).

(data not shown). These data therefore show that inactivation of α-amylase is a consequence of the formation of a stable difluoroglycosyl-enzyme intermediate at the active site of the enzyme.

Similar data were also acquired with yeast α-glucosidase inactivated by TNPDFG, and kinetic parameters for this process along with other data are shown in Table I. In this case again, a competitive inhibitor, 1-deoxyxojirimycin (45 μM, $K_i$ = 12.6 μM), was shown to protect the enzyme against inactivation, reducing the rate of inactivation in the presence of 1.6 mM TNPDFG from 0.13 min⁻¹ to 0.073 min⁻¹.

It is of interest to compare the rate reductions consequent upon introduction of the fluoride substituents at C-2 in the two cases. As can be seen in Table I, introduction of the first fluoride at C-2 reduces the glycosylation rate (from $k_{cat}/K_m$ values) some 2700-fold for α-amylase but only 80-fold for the α-glucosidase. Comparison of parameters for the trinitrophenyl difluoroglycosides with those for the 2-fluoro glycosyl fluorides reveals a 330-fold reduction for α-amylase and again an 80-fold reduction for the α-glucosidase. This reveals a much greater sensitivity of the α-amylase-catalyzed reaction to the introduction of fluoride substituents than that for α-glucosidase, indicating either a greater degree of oxocarbenium ion character at the transition state for α-amylase, or more important interactions with the 2-hydroxyl. These massive reductions in glycosylation rate constants consequent upon the introduction of two fluorines (almost 10⁶-fold for α-amylase) were also reflected in reduced deglycosylation rate constants. No reactivation of either inactivated enzyme was seen when a sample of the inactivated enzyme was dialyzed to remove excess inactivator, then incubated for up to 30 days and aliquots removed for assay.

In summary, a new class of mechanism-based inactivator of human pancreatic α-amylase and of yeast α-glucosidase has been synthesized and shown to function via the stoichiometric trapping of a covalent glycosyl-enzyme intermediate. In addition to providing powerful evidence of the commonality of mechanisms of β- and ω-glycosidases, such inhibitors should prove useful in identifying the active site nucleophiles of these and other glycosidases, and compounds of this class may well prove valuable as therapeutic agents.

Acknowledgments—We thank Yili Wang for technical assistance, Drs. Ross MacGillivray and Helene Cote for assistance with supply of enzyme, and John McCarter for helpful discussions.

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