Enzymatic Synthesis of Carbon−Fluorine Bonds

David L. Zechel,[†] Stephen P. Reid,[†] Oyekanmi Nashiru,[‡] Christoph Mayer,[†] Dominik Stoll,[‡] David L. Jakeman,[†] R. Antony J. Warren,[‡] and Stephen G. Withers*[,†]

Protein Engineering Network of Centres of Excellence and Departments of Chemistry and Microbiology University of British Columbia Vancouver, B.C., Canada V6T 1Z1

Received December 6, 2000

Halogenated secondary metabolites are ubiquitous in Nature. Over 3000 natural products containing chlorine, bromine, or iodine have been characterized to date, and scores are added to the list each year.[1] There is, however, a notable scarcity of fluorinated natural products, of which only 13 are known.[2] This may in part stem from the limited bioavailability of fluoride, which exists primarily in insoluble mineral forms.[3,4] Equally elusive is an enzymatic mechanism for fluorination, as no enzyme with fluorinating activity has yet been isolated.[2] A key aspect of the fluorination mechanism will be the nature of the fluorinating species: electrophilic, radical, or nucleophilic. The electrophilic halogenation mechanism is well-known for the haloperoxidases,[5] and a radical chlorination mechanism is thought to be involved in the biosynthesis of barbamide.[6] However, the chemical oxidation of fluoride to "F^{+}" ($E° = 2.87$ V),[7] the strongest oxidizing agent known, is infeasible and therefore these mechanisms are unlikely. The nucleophilic halogenation mechanism is rare, having only been demonstrated for the methylation of Br^-, Cl^-, and I^- by *S*-adenosylmethionine methyl transferases.[8] A nucleophilic mechanism also appears to be used by FAD/NADH dependent halogenases in the biosynthesis of pyrrolnitrin,[9] and is thought to be involved in the biosynthesis of fluoroacetate.[4]

Given the scarcity of data on nucleophilic halogenation by enzymes, particularly with fluoride, it is significant that specific active site mutants of two retaining glycosidases, *Agrobacterium* sp. β-glucosidase (Abg) and *Cellulomonas fimi* β-mannosidase (Man2A), can catalyze the formation of carbon−fluorine bonds with nucleophilic fluoride.[10] Replacement of the catalytic glutamate nucleophile in Abg (E358) or Man2A (E519) with alanine, glycine, or serine renders the usual double displacement mechanism[11] inoperable and virtually no glycosidic bond cleaving activity is detectable. However, when assayed with the appropriate 2,4-dinitrophenyl β-glycoside substrate in the presence of fluoride (2 M KF, pH 6, 25 °C), substantial glycosidic bond activity is restored (Table 1).[12] Because fluoride is acting in the place of the missing catalytic nucleophile, the product of the glycosidic bond cleaving reaction for Abg and Man2A nucleophile mutants is the corresponding α-glycosyl fluoride[13] (Scheme 1, first step) as confirmed by TLC, ^{1}H NMR, and ^{19}F NMR.[14a] In the case of Abg E358G, the turnover for carbon−fluorine bond formation exceeds 2 s^{-1}.[15] However, because these nucleophile mutants, or "glycosynthases",[10,16] also act as catalysts for oligosaccharide synthesis, the α-glycosyl fluoride product in turn acts as a glycosyl donor in a subsequent transglycosylation reaction with a second molecule 2,4-dinitrophenyl β-glycoside, thus forming new glycosidic bonds (Scheme 1, second step).[14a] The wild-type enzymes did not catalyze the formation of α- or β-glycosyl fluorides, as indicated by ^{19}F NMR, presumably due to electrostatic or steric effects.[17]

The Abg E358G and E358A mutants can also catalyze nucleophilic halogenation of 2,4-dinitrophenyl β-glucoside with chloride and bromide (2 M). Although the corresponding α-glucosyl halides are too unstable to isolate directly,[14b] transglycosylation products resulting from transfer to a second equivalent of substrate were observed by TLC and ESI-MS. A comparison of k_{cat}/K_M values for E358G and E358A (Table 1) indicates an order of halide reactivity ($F^- > Cl^- > Br^-$) opposite to that expected in aqueous solution. Although this may be the result of steric constraints in the Abg active site, this order of halide nucleophilicity has also been observed in organic solvents and the gas phase.[18] Desolvation of the halide may well occur in the active site of Abg. The reactivity order may also reflect a stabilizing "synergism"[18,19] in the halogenation transition state

* Address correspondence to this author. Phone: (604) 822-3402. Fax: (604) 822-2847. E-mail: withers@chem.ubc.ca.

† Department of Chemistry

‡ Department of Microbiology

(1) Gribble, G. W. *Chem. Soc. Rev.* **1999**, *28*, 335−346.

(2) O'Hagan, D.; Harper, D. B. *J. Fluorine Chem.* **1999**, *100*, 127−133.

(3) Cotton, F. A.; Wilkinson, G. *Advanced Inorganic Chemistry*, 5th ed.; John Wiley and Sons: New York, 1988.

(4) Harper, D. B.; O'Hagan, D. *Nat. Prod. Rep.* **1994**, 123−133.

(5) (a) Butler, A. *Coord. Chem. Rev.* **1999**, *187*, 17−35. (b) Littlechild, J. *Curr. Opin. Chem. Biol.* **1999**, *3*, 28−34. (c) Hofmann, B.; Tölzer, S.; Pelletier, I.; Altenbuchner, J.; van Pée, K.-H.; Hecht, H. J. *J. Mol. Biol.* **1998**, *279*, 889−900. (d) van Pée, K.-H. *Annu. Rev. Microbiol.* **1996**, *50*, 375−99.

(6) (a) Hartung, J. *Angew. Chem., Int. Ed.* **1999**, *38*, 1209−11. (b) Sitachitta, N.; Rossi, J.; Roberts, M. A.; Gerwick, W. H.; Fletcher, M. D.; Willis, C. L. *J. Am. Chem. Soc.* **1998**, *120*, 7131−32.

(7) *CRC Handbook of Chemistry and Physics*, 71st ed.; CRC Press: Boston, 1990−1991.

(8) Wuosmaa, A. M.; Hager, L. P. *Science* **1990**, *249*, 160−162.

(9) (a) van Pée, K.-H.; Keller, S.; Wage, T.; Wynands, I.; Schnerr, H.; Zehner, S. *Biol. Chem.* **2000**, *381*, 1−5. (b) Keller, S.; Wage, T.; Hohaus, K.; Hölzer, M.; Eichhorn, E.; van Pée, K.-H. *Angew. Chem., Int. Ed.* **2000**, *39*, 2300−2302.

(10) We first noted fluoride dependent glycosidic bond cleaving activity with Man2A nucleophile mutants (Nashiru, O.; Zechel, D. L.; Stoll, D.; Mohammadzadeh, T.; Warren, R. A. J.; Withers, S. G. *Angew. Chem., Int. Ed.* **2001**, *40*, 417−419).

(11) Zechel, D. L.; Withers, S. G. *Acc. Chem. Res.* **2000**, *33*, 11−18.

(12) Evolved halide specificity in a wild-type enzyme can be expected to produce equivalent catalysis at "physiological" concentrations of fluoride.

(13) This is likely a concerted single-displacement mechanism since glycosyl oxocarbenium ion intermediates do not exist in the presence of anions.[22,26]

(14) (a) TLC analysis of a reaction of Man2A E519S, 2,4-dinitrophenyl β-mannoside, and 1 M KF indicated the formation of α-mannosyl fluoride as the major product. ^{1}H and ^{19}F NMR spectra of the isolated, per-*O*-acetylated product agreed with that of an authentic sample: ^{1}H NMR (200 MHz, $CDCl_3$) δ 5.55 (dd, 1 H, $J = 48.3$, 1.7 Hz, H-1), 5.40−5.30 (m, 3 H, H-2,3,4), 4.29 (dd, 1 H, $J = 12.7$, 5.4 Hz, H-6ax), 4.14 (m, 2 H, H-5, H-6eq); ^{19}F NMR (188 MHz, $CDCl_3$, referenced to CF_3CO_2H) δ −62.5 (d, $J = 49.2$ Hz). α-Glucosyl fluoride could not be isolated from a preparative reaction with Abg E358S, 2,4-dinitrophenyl β-glucoside, and 2 M KF due to the more potent glycosynthase activity of this mutant, leading to its rapid consumption via transglycosylation. The rate constant for turnover of α-glucosyl fluoride with Abg E358S exceeds 1 s^{-1} (unpublished). Di- and trisaccharide products were the major products detected by TLC and ESI-MS. However, a low concentration of α-glucosyl fluoride produced by the reaction with Abg E358G was confirmed by ^{19}F NMR (282 MHz, referenced to CF_3CO_2H): δ −74.3 (dd, $J = 55.4$, 24.9 Hz,). Moreover, the analogous reaction with 2,4-dinitrophenyl β-galactoside and Abg E358G resulted in the formation of α-galactosyl fluoride as the major product. α-Galactosyl fluoride cannot donate to a second β-galactoside substrate due to the absence of a suitably positioned (equatorial) 4-hydroxyl.[16] ^{1}H and ^{19}F NMR spectra of the isolated, per-*O*-acetylated product agreed with that of an authentic sample. ^{1}H NMR (200 MHz, $CDCl_3$) δ 5.78 (dd, 1 H, $J = 2.4$, 53 Hz, H-1), 5.50 (dd, 1 H, $J = 2.9$, 1 Hz, H-4), 5.34 (dd, 1 H, $J = 3.2$, 10.7 Hz, H-3), 5.16 (ddd, 1 H, $J = 2.9$, 10.7, 23.2 Hz, H-2), 4.38 (m, 1 H, H-5), 4.11 (m, 2 H, 2 × H-6); ^{19}F NMR (188 MHz, $CDCl_3$, referenced to CF_3CO_2H) δ −75.1 (dd, $J = 53.4$, 22.9 Hz). (b) During the reaction of Abg E358G with 2,4-dinitrophenyl β-galactoside and 2 M NaCl (pD 5.6, 300 K) a small resonance was observed by ^{1}H NMR that may correspond to α-galactosyl chloride: δ 5.45 (d, $J = 3.1$ Hz, H-1, referenced to HDO). The instability of this compound precluded synthesis of a standard.

(15) The activity of a mutant glutamate dehydrogenase was enhanced 10^3-fold by fluoride fulfilling an electrostatic role (Hayden, B. M.; Dean, J. L. E.; Martin, S. R.; Engel, P. C. *Biochem. J.* **1999**, *340*, 555−560).

(16) Mayer, C.; Zechel, D. L.; Reid, S. P.; Warren, R. A. J.; Withers, S. G. *FEBS Lett.* **2000**, *466*, 40−44.

(17) The precision of chemical rescue in glycosidase mutants is well documented (Ly, H. D.; Withers, S. G. *Annu. Rev. Biochem.* **1999**, *68*, 487−522).

(18) (a) Olmstead, W. N.; Brauman, J. I. *J. Am. Chem. Soc.* **1977**, *99*, 4219−4228. (b) Parker, A. J. *J. Chem. Soc.* **1961**, 1328−1337.

(19) Pearson, R. G.; Songstad, J. *J. Org. Chem.* **1967**, *32*, 2899−2900.

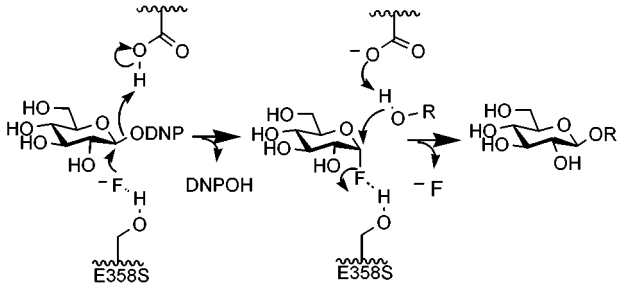
10.1021/ja005855q CCC: $20.00
Published on Web 04/13/2001

Table 1. Kinetic Parameters for Abg and Man2A Nucleophile Mutants Catalyzing Nucleophilic Halogenation of 2,4-Dinitrophenyl β-Glucoside and 2,4-Dinitrophenyl β-Mannoside, Respectively

	k_{cat} (min^{-1})[a]	K_M (mM)	k_{cat}/K_M (min^{-1}·mM^{-1})
Abg wt	5040	0.0214	235 000
+ 2 M KF	3600	0.024	150 000
Abg E358A	<0.001		
+ 2 M KF	21.5	0.131	164
+ 2 M NaCl	1.00	0.112	8.93
Abg E358G	0.08		
+ 2 M KF	131	0.157	834
+ 2 M NaCl	44.8	0.086	521
+ 2 M NaBr	11.2	0.144	77.8
+ 2 M KI	n/a[b]		
Abg E358S	<0.001		
+ 2 M KF	28.9	0.044	657
Man2A wt	27 000	0.6	45 000
+ 2 M KF	7 900	0.22	36 000
Man2A E519A	<0.008		
+ 2 M KF	20.7	0.131	158
Man2A E519S	0.0015		
+ 2 M KF	14.0	0.036	389

[a] k_{cat}, K_M apply to glycoside at 2 M halide, pH 6.0, 25 °C. [b] No activity.

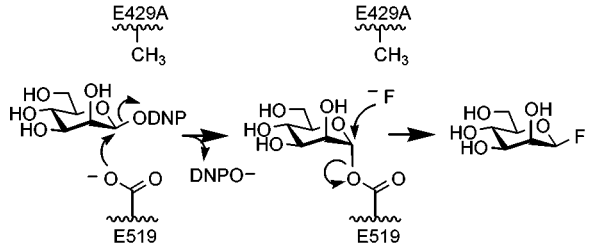
Scheme 1. Abg E358S Catalyzing Nucleophilic Fluorination of 2,4-Dinitrophenyl β-Glucoside and Subsequent Transfer of α-Glucosyl Fluoride to a Second Equivalent of Substrate (R)



between hard, weakly polarizable attacking and leaving groups (i.e. fluoride and phenolate oxygen).[20] Synergism may also account for similar reversals in halide reactivity that have been observed for the reaction of methyl glycosides[21] and α-glucosyl fluoride[22] with halides in aqueous solution.

Also noteworthy from the k_{cat}/K_M values is the fluorination activity of the Man2A and Abg serine mutants (Table 1). This activity is greater than or comparable to the fluorination activity of the glycine and alanine mutants, despite the extra bulk of the serine side chain. It is probable that this difference arises from a hydrogen bond to the serine hydroxyl group. Abg E358G may also benefit from a hydrogen bond between fluoride and Tyr 298, a residue that interacts with the E358 oxygen in the wild-type enzyme.[23] Although fluorine is, at best, a poor hydrogen bond acceptor,[24] desolvated fluoride can form some of the strongest

Scheme 2. Man2A E429A Catalyzing Nucleophilic Fluorination of the Mannosyl-Enzyme Covalent Intermediate, Forming β-D-Mannosyl Fluoride. (DNP = 2,5-dinitrophenyl)



hydrogen bonds known, with hydrogen bond energies of 30 kcal/mol realized with methanol or ethanol as partners in the gas phase.[25] Moreover, the departure of fluoride during the solvolysis of α-glucosyl fluoride is acid catalyzed,[26] even when trifluoroethanol (pK_a = 12.4) acts as the proton donor.[22,27] Likewise, a hydrogen bond formed with the departing fluoride ion in the glycosynthase transition state (Scheme 1, second step) may also explain the ~25-fold greater glycosynthase activity of Man2A and Abg serine mutants relative to the alanine mutants.[10,16]

The generality of enzymatic nucleophilic fluorination is further demonstrated by the ability of the general acid−base mutant of Man2A (E429A) to synthesize β-mannosyl fluoride. This mutant readily forms the covalent intermediate with the reactive substrate 2,5-dinitrophenyl β-mannoside (Scheme 2). Due to the absence of base catalysis in the second step, deglycosylation is slowed and the covalent intermediate accumulates, as evidenced by very low k_{cat} and K_M values (k_{cat} = 12 min^{-1}, K_M < 1 μM). However, fluoride (1 M KF) can attack the anomeric carbon of the covalent intermediate, resulting in faster turnover of the enzyme (k_{cat} = 87 min^{-1}) and a corresponding rise in K_M (7.7 μM), as well as the formation of β-mannosyl fluoride. The β-mannosyl fluoride product could not be isolated and is not expected to accumulate as it is also a good substrate for Man2A.[28] Nevertheless, while monitoring the reaction directly by ^{19}F NMR spectroscopy a resonance corresponding to a low steady-state concentration of β-mannosyl fluoride was observed.[29]

The catalysis of carbon−fluorine bond formation by mutants of two unrelated glycosidases, each using a nucleophilic fluorination mechanism, demonstrates the feasibility of such a mechanism occurring in Nature.[30] Indeed, it is particularly interesting that one of the known fluorinated natural products, nucleocidin,[2] is itself a glycosyl fluoride and may well be formed by an analogous mechanism. As suggested by this work, catalytic features of nucleophilic fluorination may involve hydrogen bonding and desolvation of the anion. This work also raises the possibility of engineering other enzymes to catalyze nucleophilic fluorination of medicinally significant compounds.

Acknowledgment. We thank the Natural Sciences and Engineering Research Council of Canada (NSERC) and the Protein Engineering Network of Centres of Excellence (PENCE) for financial support.

JA005855Q

(20) This is consistent with the halide reactivity order observed for the *S*-adenosylmethionine methyl transferase reaction ($I^- \gg Br^- > Cl^-$), which involves a "soft" leaving group, sulfur.[8] Fluoride is not a substrate. However, halide specificity may have evolved in these enzymes.

(21) Bennet, A. J.; Sinnott, M. L. *J. Am. Chem. Soc.* **1986**, *108*, 7287−94.
(22) Banait, N. S.; Jencks, W. P. *J. Am. Chem. Soc.* **1991**, *113*, 7951−58.
(23) (a) Gebler, J. C.; Trimbur, D. E.; Warren, R. A. J.; Aebersold, R.; Namchuk, M.; Withers, S. G. *Biochemistry* **1995**, *34*, 14547−53. (b) Hakulinen, N.; Paavilainen, S.; Korpela, T.; Rouvinen, J. *J. Struct. Biol.* **2000**, *129*, 69−79.
(24) Dunitz, J. D.; Taylor, R. *Chem. Eur. J.* **1997**, *3*, 89−98.
(25) Hibbert, F.; Emsley, J. *Adv. Phys. Org. Chem.* **1990**, *26*, 255−379.
(26) Banait, N. S.; Jencks, W. P. *J. Am. Chem. Soc.* **1991**, *113*, 7958−61.
(27) Sinnott, M. L.; Jencks, W. P. *J. Am. Chem. Soc.* **1980**, *102*, 2026−32.
(28) Stoll, D.; He, S.; Withers, S. G.; Warren, R. A. J. *Biochem. J.* **2000**, *351*, 833−8.

(29) Man2A E429A (4 μM) was reacted with 2,5-dinitrophenyl β-mannoside (23 mM) in 1 M KF, pH 7, at 300 K. After 20 min an apparent doublet was observed by ^{19}F NMR (282 MHz, referenced to $CFCl_3$): δ −146.7 (d, J = 49 Hz). This agreed with an authentic sample of β-mannosyl fluoride.

(30) In fact, there is strong evidence that haloperoxidases may not be involved in the biosynthesis of most halometabolites and that nucleophilic halogenation may be Nature's preferred biosynthetic mechanism.[5d,9a]