Contribution of the active site aspartic acid to catalysis in the bacterial neuraminidase from Micromonospora viridifaciens. FEBS Lett

ARTICLE *in* FEBS LETTERS · DECEMBER 2004

Impact Factor: 3.17 · DOI: 10.1016/j.febslet.2004.10.016 · Source: PubMed

5 AUTHORS, INCLUDING:

Simon Newstead
University of Oxford
45 PUBLICATIONS **1,264** CITATIONS
SEE PROFILE

Garry L Taylor
University of St Andrews
123 PUBLICATIONS **4,619** CITATIONS
SEE PROFILE

Contribution of the active site aspartic acid to catalysis in the bacterial neuraminidase from *Micromonospora viridifaciens*

Jacqueline N. Watson[a], Simon Newstead[b], Veedeeta Dookhun[a], Garry Taylor[b,*], Andrew J. Bennet[a,*]

[a] *Department of Chemistry, Simon Fraser University, 8888 University Drive, Burnaby, BC, Canada V5A 1S6*
[b] *Centre for Biomolecular Science, University of St Andrews, St Andrews, Fife KY16 9ST, Scotland, UK*

Received 19 August 2004; revised 30 September 2004; accepted 6 October 2004

Available online 18 October 2004

Edited by Hans-Dieter Klenk

Abstract A recombinant D92G mutant sialidase from *Micromonospora viridifaciens* has been cloned, expressed and purified. Kinetic studies reveal that the replacement of the conserved aspartic acid with glycine results in a catalytically competent retaining sialidase that possesses significant activity against activated substrates. The contribution of this aspartate residue to the free energy of hydrolysis for natural substrates is greater than 19 kJ/mol. The three dimensional structure of the D92G mutant shows that the removal of aspartic acid 92 causes no significant re-arrangement of the active site, and that an ordered water molecule substitutes for the carboxylate group of D92.

Keywords: Neuraminidase; Sialidase; Catalytic mechanism; Mutagenesis; *Micromonospora viridifaciens*

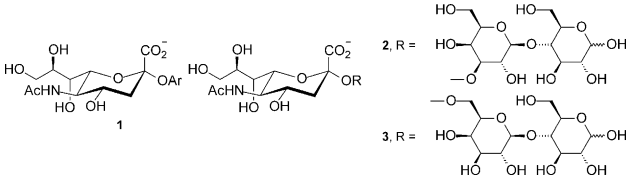
1. Introduction

Sialidases (*N*-acetylneuraminosyl glycohydrolase, neuraminidase, EC 3.2.1.18) belong to a family of exoglycosidases [1] and some of these enzymes are involved in the pathogenesis of several diseases [2,3]. Sialidases and *trans*-sialidases contain seven strictly conserved amino acids [4–10], namely: (a) an arginine triad which binds to the carboxylate group of the substrate via electrostatic interactions; (b) a distal glutamate residue which forms a salt bridge with one of the conserved arginine residues; and (c) three residues that are important for catalysis, a tyrosine–glutamic acid dyad and an aspartic acid.

All known natural sialidases catalyze the hydrolysis of substrates with retention of anomeric configuration [11], an observation that is compatible with this family of enzymes operating via a standard double displacement mechanism [11–16]. Recently, Watson et al. [17] showed that three separate tyrosine mutants of the enzyme from *Micromonospora viridifaciens* are inverting sialidases. Such an observation is evidence for the conserved tyrosine residue being the catalytic nucleophile in typical sialidases and that water acts as the nucleophile in the inverting mutant enzymes by occupying the "hole" created when the tyrosine is replaced by a smaller amino acid. Watts et al. [18,19] also concluded that tyrosine is the nucleophile in *trans*-sialidases by showing that a mutant enzyme becomes covalently modified on the active-site tyrosine during turnover of a fluorinated substrate analog. Accordingly, the presumed role of the glutamate residue of the catalytic dyad is to facilitate nucleophilic attack of the tyrosine by acting as a general-base.

With regard to the third catalytic residue, namely the conserved aspartic acid, it has been proposed that its role is to act as a general acid catalyst [20]. The results from previous mutagenic studies on this aspartic acid residue led to the conclusion that it is important for catalysis, but its specific energetic contribution to the catalysis was not delineated [21–23].

The present paper details a series of kinetic and product studies that were performed on the D92G mutant of *M. viridifaciens* sialidase using a variety of substituted aryl α-D-*N*-acetylneuraminides (**1**) and two natural substrate analogs, 3′-sialyllactose (**2**) and 6′-sialyllactose (**3**). In addition, in order to understand any structural implications mutating the aspartic acid to a glycine might have on the integrity of the active site, the three dimensional structures of the D92G mutant in the apo form and complexed with the inhibitor 2-deoxy-2,3-dehydro-N-acetylneuraminic acid (Neu5Ac2en, DANA), a compound believed to mimic the reaction transition state, were studied using X-ray crystallography.



2. Materials and methods

2.1. Materials

All restriction endonucleases and DNA modification enzymes were purchased from Gibco BRL or New England BioLabs (Beverly, MA). All DNA manipulations were carried out according to standard

* Corresponding authors. Fax: +44-1334-462595 (G. Taylor), +1-604-291-3765 (A.J. Bennet).
E-mail addresses: glt2@st-and.ac.uk (G. Taylor), bennet@sfu.ca (A.J. Bennet).

Abbreviations: MU-αNeu5Ac, 4-methylumbelliferyl α-D-*N*-acetylneuraminide

0014-5793/$22.00
doi:10.1016/j.febslet.2004.10.016

procedures [24]. All chemicals were of analytical grade or better and were purchased from Sigma–Aldrich unless otherwise noted.

Site-directed mutagenesis – Mutagenesis was performed according to the previously described strategy, using the pJWHS2 plasmid as the template for the sialidase gene [17]. Reverse mutagenic primers were D92G-R′ (5′-GGGCGCGCCGATACCGGTCG-3′), D92A-R′ (5′-GGGCGCGGCGATACCGGTCG-3′), and D92S-R′ (5′-CGGGCGCGGAGATACCGGTCG-3′) (codon substitution is underlined). Forward mutagenic primer sequences were D92G-F′ (5′-GTATCGGCGCGCCCGGCCC-3′), D92A-F′ (5′-GTATCGCCGCGCCCGGCCC-3′), and D92S-F′ (5′-GT-ATCTCCGCGCCCGGCCC-3′). Restriction and DNA sequence analysis was used to confirm the three separate D92 mutations, as well as to ensure that spurious mutations had not occurred during DNA manipulation.

2.2. Enzyme purification and kinetics

The sialidase variants D92G, D92A and D92S were expressed in a manner identical to that reported for the wild-type enzyme, and purification of the D92G mutant and all kinetic experiments followed the previously described protocols [17].

2.3. Crystallization and X-ray data collection

The purified protein was concentrated to 20 mg ml^{-1} in 20 mM sodium phosphate, pH 7.0, and crystallized using the sitting drop vapor diffusion method. Crystals appeared within one week at 293 K in drops composed of 2 μl protein solution plus 2 μl of mother liquor containing 13% (w/w) PEG 3350 and 0.2 M ammonium citrate. The complex with Neu5Ac2en was obtained by soaking the crystals in 20 mM Neu5Ac2en for 2 h at 293 K. Crystals were cryoprotected with 10% w/v glycerol in the crystallization buffer for one minute prior to flash freezing in a nitrogen stream at 100 K. Diffraction data were collected on the ESRF beamline ID14.2, for the apo structure, and on a Rigaku/MSC MircoMax 007 rotating anode generator, for the Neu5Ac2en complex. All data were processed using MOSFLM [25] and other programs from the CCP4 suite [26]. The crystals belong to space group $P2_12_12_1$, with one molecule in the asymmetric unit. Cell dimensions were $a = 46.6$ Å, $b = 110.35$ Å, and $c = 141.88$ Å for the unliganded enzyme, and $a = 46.6$ Å, $b = 111.61$ Å, and $c = 143.87$ Å for the Neu5Ac2en complex. Data statistics are given in Table 1.

2.4. Structure solution

The starting model for crystallographic refinement was the 2.5 Å structure of the *M. viridifaciens* sialidase reported previously [7], PDB code 1EUU, which was in a monoclinic $P2_1$ unit cell. A molecular replacement solution in the new orthorhombic cell for the apo structure was found using the complete model with AMoRe [27]. Rigid body refinement of the three domains (residues 47–402, 403–502 and 503–647) was followed by restrained refinement using REFMAC5 [28], with iterative cycles of model building in the program O. Solvent molecules were added automatically using the program ARP/wARP [29] and visually inspected for correct placement in O [30]. The final crystallographic statistics are given in Table 1. The apo structure revealed lactose bound to the galactose-binding domain via its galactose, and the active site occupied by a citrate and glycerol. The Neu5Ac2en complex revealed the inhibitor bound to the active site and the galactose-binding domain occupied by an ordered galactose.

Atomic coordinates have been deposited with the Protein Databank with codes 1W8O and 1W8N for the D92G apo and D92G Neu5Ac2en complex, respectively.

3. Results and discussion

3.1. Mechanism utilized by mutants of the conserved aspartic acid

Three separate mutants of the conserved aspartic acid of the *M. viridifaciens* sialidase were produced recombinantly in an *Escherichia coli* expression system. These variants (D92G, D92A and D92S) were expressed at levels similar to the wild-type enzyme. Moreover, the supernatants for the expression of all three mutants displayed a comparable catalytic activity to that of the native enzyme using the substrate MU-αNeu5Ac. The purified D92G mutant was selected for detailed mechanistic and structural investigation because it was the most active mutant. The thermostability profiles of this mutant and the wild-type enzyme are shown in Fig. 1.

Table 1
Data collection and crystallographic analysis

Crystal	D92G	D92G+Neu5Ac2en
X-ray source	ESRF ID14-2	in-house
Measured reflections	1 060 418	175 360
Unique reflections	82 427	44 772
Maximum resolution (Å)	1.70 (1.79–1.70)	2.10 (2.20–2.10)
Completeness (%)	92 (72)	89 (88)
R-merge	0.070 (0.32)	0.068 (0.30)
Refinement		
Resolution range (Å)	87–1.70	87–2.10
R-factor	0.163	0.166
R-free	0.198	0.219
RMS bond lengths (Å)	0.02	0.021
RMS bond angles (°)	1.84	1.77
Number of proteins atoms	4568	4555
Number of waters	711	519
Number of ligand atoms	56	33

Figures in parentheses refer to the highest resolution shell of data.

Fig. 2 shows a graphical representation of the time course for D92G mutant-catalyzed hydrolysis of MU-αNeu5Ac monitored by ^1H NMR spectroscopy. Thus, the D92G mutant and the wild-type [17] sialidases display homologous mechanistic traits in that both catalyze substrate hydrolysis with retention of anomeric configuration. Therefore, it can be concluded that the D92G mutant-catalyzed reaction is operating via a double displacement mechanism in which the conserved tyrosine residue (Y370) is acting nucleophilically. Also, the D92G mutant is remarkably active, for example, using the fluorogenic substrate MU-αNeu5Ac, its measured k_{cat}/K_m value is 82% of that for the wild-type enzyme.

3.2. Contributions of the aspartic acid residue to catalysis

An obvious question that needs answering is: what is the function of the aspartic acid if its removal results in a catalytically active enzyme? The data summarized in Table 2 show that a pronounced change in relative catalytic activity occurs between the wild-type and the D92G mutant sialidases

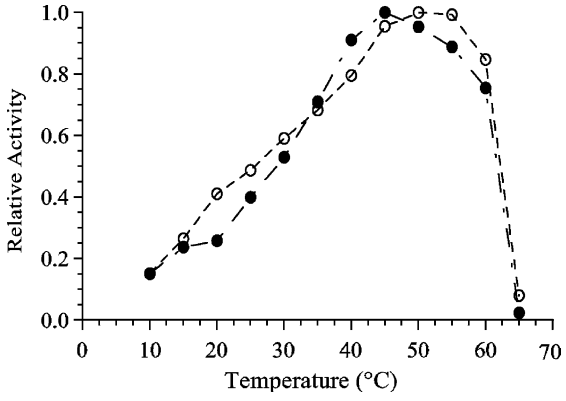


Fig. 1. Effect of temperature on the relative rates of sialidase-catalyzed hydrolysis of MU-αNeu5Ac at pH 5.25 for the wild-type enzyme (○) and D92G mutant (●). Data for wild-type reproduced with permission of the American Chemical Society.

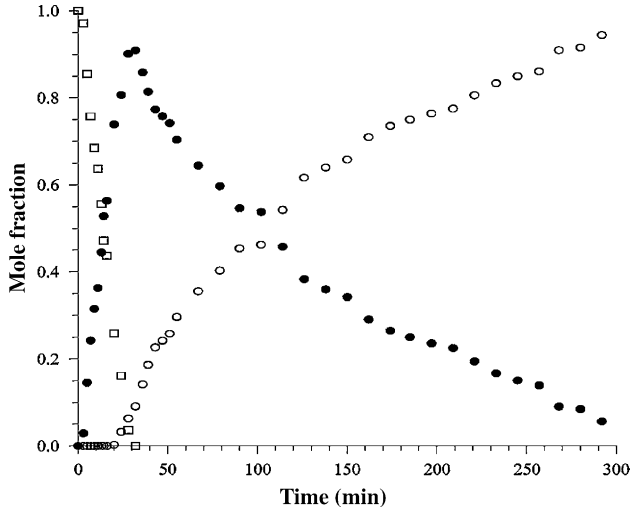


Fig. 2. Mole fractions of substrate and products monitored over time for the D92G mutant-catalyzed hydrolysis of MU-αNeu5Ac. Values were calculated using the relative integrals of the H_{3eq} signals of MU-αNeu5Ac (□), αNeu5Ac (●) and βNeu5Ac (○).

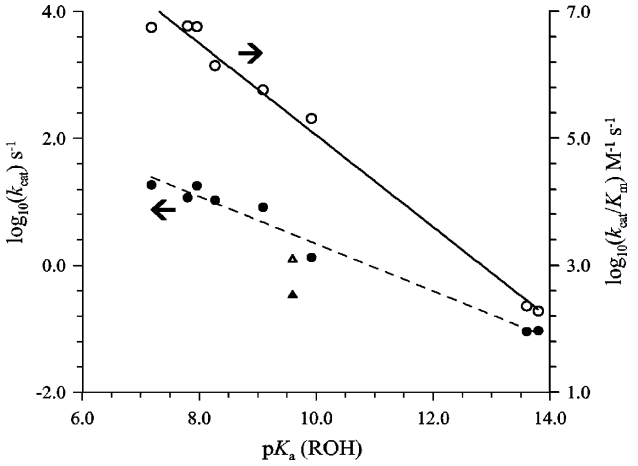


Fig. 3. Brønsted plots. Effect of leaving group ability on k_{cat} (●) and k_{cat}/K_m (○) for the D92G mutant sialidase at 37 °C and a pH of 5.25. Leaving group ability represented as pK_a (BH^+) is as follows: 4-nitrophenol (7.18); 4-methylumbelliferone (7.80); 4-cyanophenol (7.96); 3-nitrophenol (8.27); 3-chlorophenol (9.09); 3-methoxyphenol (9.60); phenol (9.92); and lactose (3′-OH (~13.6) and 6′-OH (~13.8)). The experimental points for the enzyme-catalyzed hydrolysis of the 3-methoxyphenyl substrate (▲ k_{cat}; and △ k_{cat}/K_m) are omitted from the displayed best fit lines.

on altering the leaving group from 4-nitrophenol to lactose. In detail, Fig. 3 displays the Brønsted plots for the D92G mutant sialidase-catalyzed hydrolysis reactions. The derived β_{lg} parameters are -0.37 ± 0.02 and -0.72 ± 0.03 for the kinetic terms k_{cat} and k_{cat}/K_m, respectively. For the wild-type sialidase-catalyzed reaction, k_{cat} is limited by a step(s) that occurs after cleavage of the glycosidic bond [17], a similar finding has been reported for type A influenza virus sialidase [31], and this step likely involves a conformational change [32]. Substitution of the conserved aspartic acid by a glycine residue results in glycosidic bond cleavage becoming partially rate-limiting for k_{cat}. That is, the first-order rate constants for reaction of the Michaelis complex (k_{cat}) are influenced by the leaving group ability ($\beta_{lg} = -0.37$). As a result, it can be concluded that the transition state for D92G mutant-catalyzed cleavage of the glycosidic bond in natural substrate analogs (**2** and **3**) is at least 19 kJ/mol higher in energy ($\Delta\Delta G^{\ddagger}$ at 37 °C) than the corresponding transition state for the wild-type sialidase reaction. A similar conclusion can be made concerning the kinetic parameter k_{cat}/K_m, where the β_{lg} value increases from −0.30 (wild-type) to −0.72 (D92G). In other words, the conserved aspartic acid residue displays a greater catalytic effect when the substrate possesses a bad leaving group, such as lactose, one of the natural aglycons. It is expected that a similar effect would be observed with all natural sialosides, including glycolipids and glycoproteins, given that

Table 2
Relative catalytic activity of the wild-type and the D92G mutant sialidases

Leaving group	Relative k_{cat}[a]	Relative k_{cat}/K_m[b]
4-Nitrophenol	7.8	1.5
4-Methylumbelliferone	4.4	1.2
3′-Lactose	1.5×10^3	1.6×10^3
6′-Lactose	2.3×10^3	2.6×10^2

[a] Equals k_{cat}(wild-type)/k_{cat}(D92G).
[b] Equals k_{cat}/K_m(wild-type)/k_{cat}/K_m(D92G).

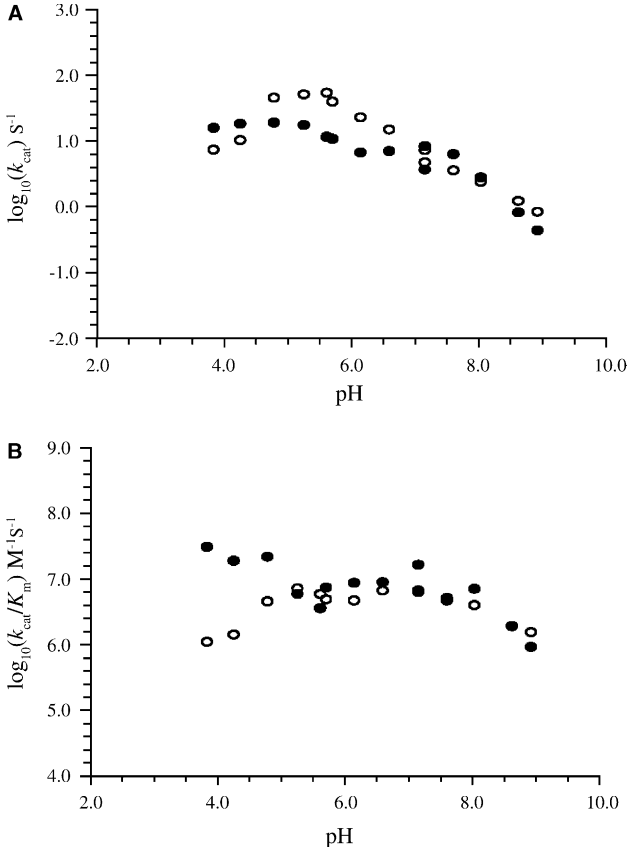


Fig. 4. Effect of pH on kinetic parameters for wild-type (○) and mutant D92G (●). (A) The pH dependence of k_{cat} and (B) the pH dependence of k_{cat}/K_M. All kinetic parameters were determined at 37 °C, using MU-αNeu5Ac as the substrate. Data for wild-type reproduced with permission of the American Chemical Society.

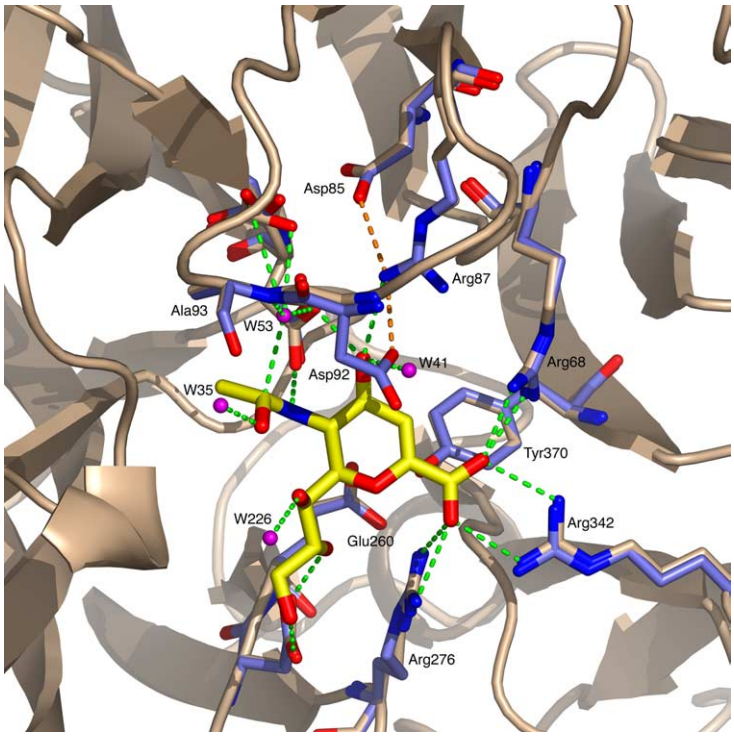


Fig. 5. Superimposition of the active site residues of wild-type catalytic domain with Neu5Ac2en (PDB 1EUS) and the D92G-Neu5Ac2en complex. Residues of the wild-type structure are shown in slate and of the mutant in wheat. The hydrogen bonding interactions are drawn as green dotted lines; the interaction of Asp92 with Asp85 is shown in orange.

the terminal residue in the aglycon is always a carbohydrate unit.

3.3. Effect of pH on the catalytic activity of the D92G mutant

The pH-rate profiles for the catalyzed hydrolysis of MU-αNeu5Ac by the D92G mutant and wild-type sialidases are strikingly similar at all pH values above 5 (Fig. 4). These observations are consistent with the phenoxide leaving group requiring little acid-catalysis for departure [33]. However, at low pH both the k_{cat} and k_{cat}/K_m values for the D92G mutant-catalyzed hydrolysis of MU-αNeu5Ac level off, while for the wild-type reactions the corresponding rate constants decrease (Fig. 4). Thus, it appears that protonation of an enzymatic residue slows down the rate-limiting step for the wild-type sialidase, which is presumed to be a conformational change, yet the D92G mutant enzyme displays no evidence for such a titration.

3.4. Crystal structure of the D92G mutant

The crystal structure of the D92G mutant shows that the substitution of the conserved aspartic acid to a more flexible glycine caused no significant perturbation of the active site (rms fit of 0.43 Å for Cα 47–402 of the catalytic domain in comparison to the wild type structure, pdb code 1EUU), and in particular, no movement of the loop that contains D92. Interestingly, the *M. viridifaciens* sialidase is one of the only two bacterial sialidases where this aspartic acid forms a direct hydrogen bond to the O4 of the ligand (2.7 Å), the other one being *Vibrio cholerae* sialidase whose equivalent aspartic acid is D62 (pdb code 1KIT). In the wild-type *M. viridifaciens* structure, D92 also interacts with NH1 and NH2 of R87 (2.7 and 2.8 Å). In the D92G-Neu5Ac2en complex, there is no change in the position of the ligand in comparison with the wild-type catalytic domain structure, pdb code 1EUS. However, as can be seen in Fig. 5, in the D92G structure a water molecule (W41) sits at a point between the oxygens of the carboxylate group of D92 in the wild-type structure, and forms hydrogen bonds with O4 of Neu5Ac2en (3.2 Å) and NH1 of R87 (2.9 Å).

Given the similarity of the crystallographic models (Fig. 5), it is not readily apparent what structural change gives rise to the different kinetic behavior at low pH values. However, it does appear that removal of aspartic acid 92 could lower the pK_a of aspartic acid 85, since they are communicating with each other via arginine 87 (Fig. 5). This hypothesis can be tested by making mutations at these non-conserved H-bonding residues.

Acknowledgements: A.J.B. is supported by the Natural Sciences and Engineering Research Council of Canada. J.N.W and S.N were supported by studentships from the Science Council of British Columbia and the UK Biology and Biotechnology Science Research Council, respectively.

References

[1] Saito, M. and Yu, R.K. (1995) in: Biology of the Sialic Acids (Rosenberg, A., Ed.), pp. 261–313, Plenum Press, New York.
[2] Corfield, T. (1993) Glycobiology 2, 509–521.
[3] Taylor, G. (1996) Curr. Opin. Struct. Biol. 6, 830–837.
[4] Varghese, J.N., Laver, W.G. and Colman, P.M. (1983) Nature (London) 303, 35–40.
[5] Crennell, S.J., Garman, E.F., Laver, W.G., Vimr, E.R. and Taylor, G.L. (1993) Proc. Natl. Acad. Sci. USA 90, 9852–9856.
[6] Crennell, S., Garman, E., Laver, G., Vimr, E. and Taylor, G. (1994) Structure 2, 535–544.
[7] Gaskell, A., Crennell, S. and Taylor, G. (1995) Structure 3, 1197–1205.

[8] Luo, Y., Li, S.C., Chou, M.Y., Li, Y.T. and Luo, M. (1998) Structure 6, 521–530.
[9] Crennell, S., Takimoto, T., Portner, A. and Taylor, G. (2000) Nat. Struct. Biol. 7, 1068–1074.
[10] Buschiazzo, A., Amaya, M.F., Cremona, M.L., Frasch, A.C. and Alzari, P.M. (2002) Mol. Cell 10, 757–768.
[11] Davies, G., Sinnott, M.L. and Withers, S.G. (1998) in: Comprehensive Biological Catalysis (Sinnott, M.L., Ed.), pp. 119–209, Academic Press, San Diego.
[12] Koshland Jr., D.E. (1953) Biol. Rev. 28, 416–436.
[13] Sinnott, M.L. (1990) Chem. Rev. 90, 1171–1202.
[14] McCarter, J.D. and Withers, S.G. (1994) Curr. Opin. Struct. Biol. 4, 885–892.
[15] Zechel, D.L. and Withers, S.G. (2000) Acc. Chem. Res. 33, 11–18.
[16] Vasella, A., Davies, G.J. and Böhm, M. (2002) Curr. Opin. Chem. Biol. 6, 619–629.
[17] Watson, J.N., Dookhun, V., Borgford, T.J. and Bennet, A.J. (2003) Biochemistry 42, 12682–12690.
[18] Watts, A.G., Damager, I., Amaya, M.L., Buschiazzo, A., Alzari, P., Frasch, A.C. and Withers, S.G. (2003) J. Am. Chem. Soc. 125, 7532–7533.
[19] Amaya, M.F., Watts, A.G., Damager, T., Wehenkel, A., Nguyen, T., Buschiazzo, A., Paris, G., Frasch, A.C., Withers, S.G. and Alzari, P.M. (2004) Structure 12, 775–784.
[20] Varghese, J.N. and Colman, P.M. (1991) J. Mol. Biol. 221, 473–486.
[21] Ghate, A.A. and Air, G.M. (1998) Eur. J. Biochem. 58, 320–331.
[22] Chien, C.H., Shann, Y.J. and Sheu, S.Y. (1996) Enzyme Microb.Technol. 19, 267–276.
[23] Kleineidam, R.G., Kruse, S., Roggentin, P. and Schauer, R. (2001) Biol. Chem. 382, 313–319.
[24] Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[25] Leslie, A.G.W. (1992) Joint CCP4+ESF-EAMCB Bewsletter on Protein Crystallography, No. 26.
[26] Bailey, S. (1994) Acta Crystallogr. D 50, 760–763.
[27] Navaza, J. (1994) Acta Crystallogr. A 50, 157–163.
[28] Murshudov, G.N., Vagin, A.A. and Dodson, E.J. (1997) Acta Crystallogr. D 53, 240–255.
[29] Lamzin, V.S. and Wilson, K.S. (1993) Acta Crystallogr. D 49, 129–147.
[30] Jones, T.A., Zou, J.Y., Cowan, S.W. and Kjeldgaard, M. (1991) Acta Crystallogr. A 47, 110–119.
[31] Chong, A.K.J., Pegg, M.S., Taylor, N.R. and von Itzstein, M. (1992) Eur. J. Biochem. 207, 335–343.
[32] Guo, X., Laver, W.G., Vimr, E. and Sinnott, M.L. (1994) J. Am. Chem. Soc. 116, 5572–5578.
[33] Guo, X. and Sinnott, M.L. (1993) Biochem. J. 294, 653–656.