SUPPLEMENTAL INFORMATION

Determination of Kinetic Mechanism

The mechanism of a multi-substrate enzyme is designated as either ping-pong if the reaction proceeds with the release of one or more products prior to the association of all substrates, or as ternary complex (or sequential) if all substrates bind to the enzyme prior to the formation of the first product. Sequential reactions are further designated as being either random if there is no obligatory order of substrate association and product release or as ordered if the substrates bind to and products are released from the enzyme in a defined order. A special case of the ordered mechanism is called the Theorell-Chance mechanism in which there is an obligatory order of substrate association and product release without the accumulation of the ternary complex under the reaction conditions. Schematic representations of these four basic types of kinetic mechanism are shown in Supplemental Fig. S1 using the notation developed by Cleland (1).

A further distinction of kinetic mechanism depends on the effect that substrate association and product release on the overall observed rate of reaction. Reactions are termed rapid equilibrium if the chemical steps are slower than all substrate association and product release steps or steady-state if the rates of substrate association and product release contribute to the overall rate of reaction.

Distinguishing Between a Sequential or Ping-Pong Mechanism

The rate equations for steady-state ping-pong and ordered Bi Bi mechanisms are shown in double reciprocal form in equations 1 and 2, respectively. In the absence of products, the equation for the Theorell-Chance "hit and run" Bi Bi mechanism is identical to that of the steady-state ordered Bi Bi mechanism.

$$\frac{1}{v_0} = \frac{K_m^A}{V_{\max}[A]} + \frac{K_m^B}{V_{\max}[B]} + \frac{1}{V_{\max}} \qquad \text{(Equation 1)}$$

$$\frac{1}{v_0} = \frac{1}{V_{\max}} + \frac{K_m^A}{V_{\max}[A]} + \frac{K_m^B}{V_{\max}[B]} + \frac{K_S^A K_m^B}{V_{\max}[A][B]} \qquad \text{(Equation 2)}$$

$$\frac{1}{v_0} = \frac{1}{V_{\max}} + \frac{K_S^A K_m^B}{V_{\max} K_S^B[A]} + \frac{K_m^B}{V_{\max}[B]} + \frac{K_S^A K_m^B}{V_{\max}[A][B]} \qquad \text{(Equation 3)}$$

The steady-state random Bi Bi mechanism is exquisitely complicated with 37 additional terms in the denominator compared to the analogous ordered mechanism (2). In many cases for random Bi Bi mechanisms, both substrates and both products are found to be in rapid and independent equilibrium with

the enzyme, making the interconversion of EAB to EPQ rate limiting. In this special case, the mechanism is called rapid equilibrium random Bi Bi and the rate equation reduces to that shown in equation 3 in double reciprocal form.

Inspection of the above equations indicates that a ping-pong mechanism can be readily distinguished from a sequential mechanism by plotting $1/v_o$ versus 1/[A] at various constant concentrations of substrate B. For a ping-pong mechanism, such a plot will yield a series of straight lines for which the slopes are equal to (K_m^{A}/V_{max}) and the intercepts are equal to $(K_m^{B}/V_{max}[B]) + (1/V_{max})$. Because the slope of each of the lines is not dependent on the concentration of substrate B, the resulting lines will be a family of parallel lines. Such a series of parallel lines is diagnostic of a ping-pong Bi Bi mechanism. In contrast, inspection of equations 2 and 3 indicates that a plot of $1/v_o$ versus 1/[A] at various constant concentrations of substrate B will yield families of straight lines for which the slope of each line is dependent on the concentration of substrate B. Double reciprocal plots for sequential mechanisms yield a family of straight lines that intersect to the left of the $1/v_o$ axis.

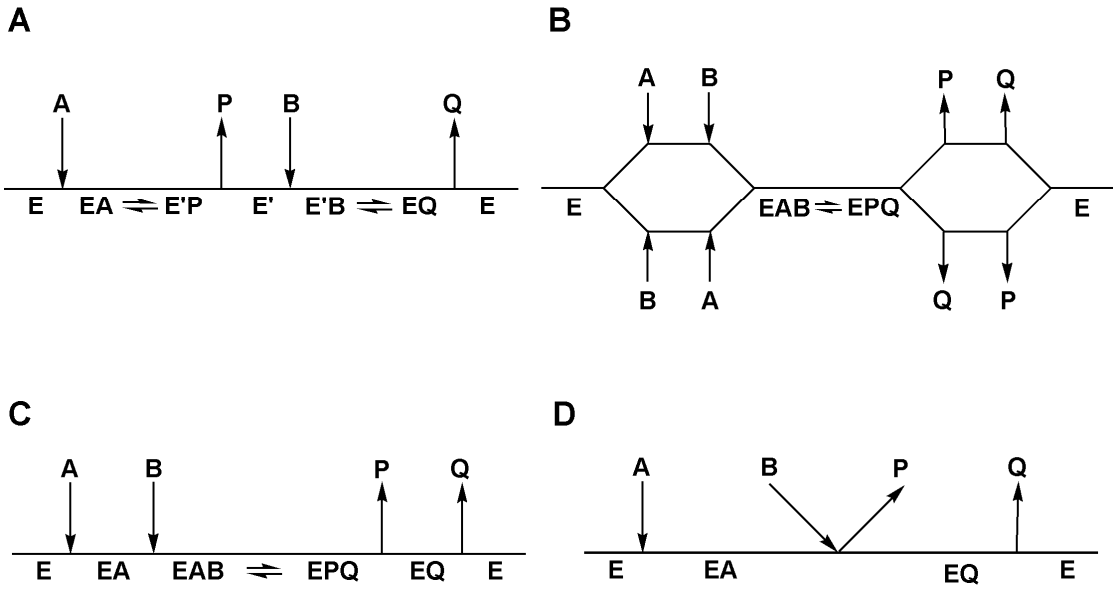

FIGURE S1. **Cleland notation schematic representations of ping-pong (A), Random sequential (B), ordered sequential (C), and Theorell-Chance (D) Bi Bi kinetic mechanisms.**

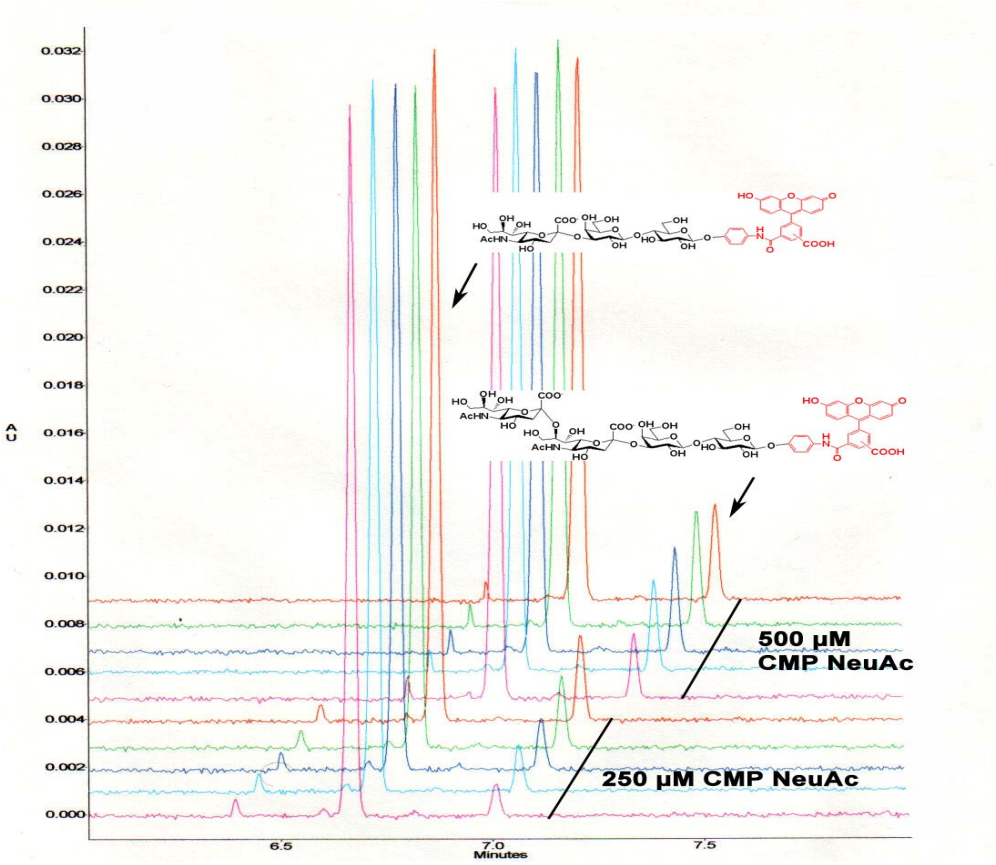

FIGURE S2. **Representative CE data used to monitor sialyltransferase Cst-II activity.**
Product formation was determined based on percent conversion of FCHASE-3'-sialyl lactose acceptor substrate using the peak areas of starting material and product. Initial rates were determined using 24 µg/ml Cst-II, various constant concentrations of CMP (0, 100, 250, 500, and 1000 µM), 500 µM FCHASE-3'-sialyl lactose, and varied concentrations of CMP-Neu5Ac (50, 100, 250, and 500 µM).

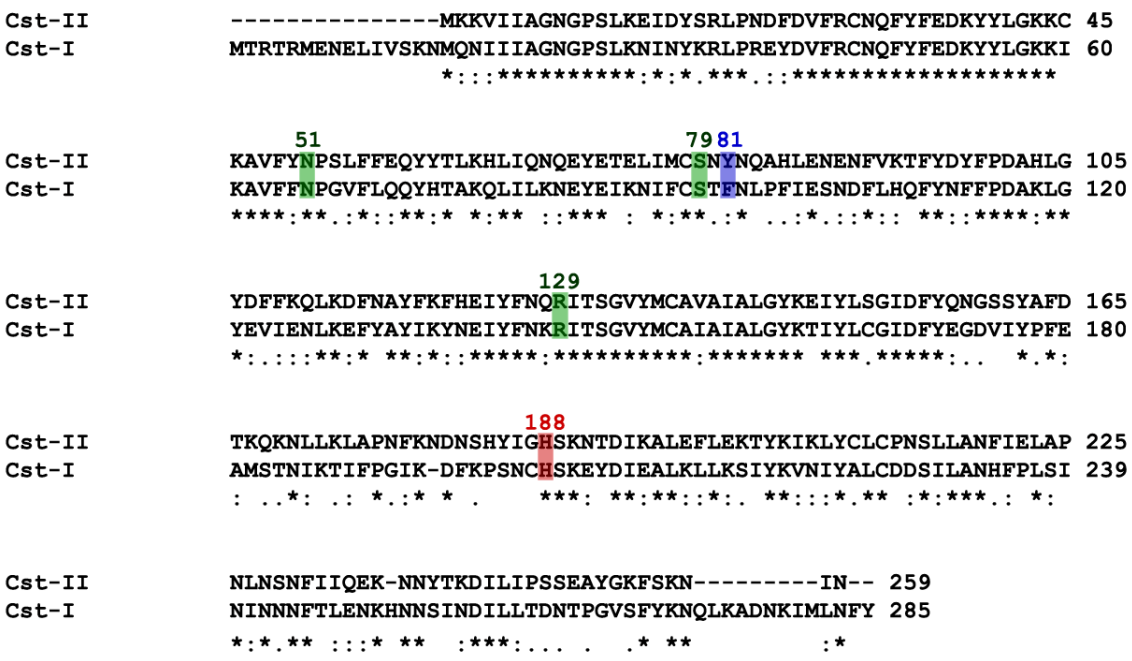

FIGURE S3. **Sequence alignment between monofunctional (Cst-I) and bifunctional (Cst-II) sialyltransferases.**

The alignment is displayed with the following symbols denoting the degree of conservation observed in amino acid sequences ("*****", identical; ":", conserved substitution; ".", semi-conserved substitution). The sequence numbering of the bifunctional Cst-II is shown. Conserved catalytic base (His-188) is highlighted in *red*, and conserved acceptor-binding residues are highlighted in *green*. Tyr-81 residue of Cst-II and its ortholog in Cst-I (Phe-96) are highlighted in *blue*. Sequence alignment was calculated using ClustalW (3).

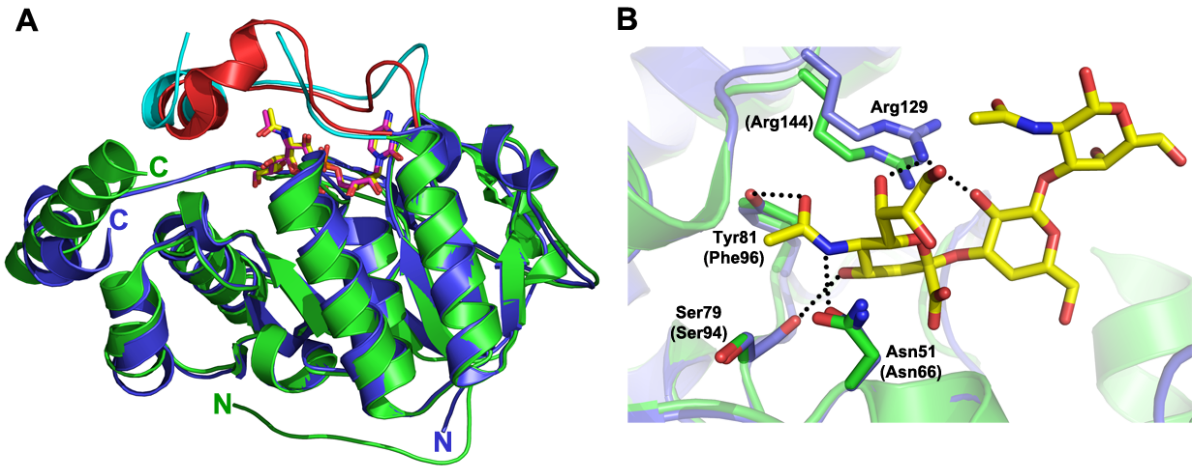

FIGURE S4. **Structural alignment between Cst-I and Cst-II.**

(A) Tertiary structure of Cst-I and Cst-II are shown in *green* and *blue*, respectively, with the N- and C-termini. Lid domains of Cst-I and Cst-II are colored in *cyan* and *red*, respectively. Carbon atoms of nonhydrolyzable donor analog (CMP-3FNeu5Ac) for Cst-I and Cst-II are colored in *yellow* and *magenta*, respectively. (B) Comparison of the acceptor-binding site of Cst-II with Cst-I. Essential acceptor-binding residues of Cst-I and Cst-II are shown in *green* and *blue*, respectively. Residue labels for Cst-I are shown in brackets. Dotted lines indicate hydrogen bonding observed in Cst-II acceptor-binding site. Carbon atoms are shown in *yellow* for the trisaccharide acceptor (Neu5Ac-α-2,3-Gal-β-1,3-GalNAc). Non-carbon atoms are colored according to atom type (nitrogen, *blue*; oxygen, *red*; phosphorus, *orange*; fluorine, *silver*).

TABLE S1. Expected product inhibition patterns for selected Bi Bi enzyme mechanisms.

Mechanism	Product Inhibitor	Varying A		Varying B	
		Unsat B	Sat B	Unsat A	Sat A
Ping-Pong Bi Bi	P	Mixed	-	Comp	Comp
	Q	Comp	Comp	Mixed	-
Steady-State Ordered Bi Bi	P	Mixed	Uncomp	Mixed	Mixed
	Q	Comp	Comp	Mixed	-
Steady-State Iso-Ordered Bi Bi	P	Mixed	Uncomp	Mixed	Mixed
	Q	Mixed	Mixed	Mixed	Uncomp
Steady-State Theorell-Chance	P	Mixed	-	Comp	Comp
	Q	Comp	Comp	Mixed	-
Rapid Equilibrium Random Bi Bi	P	Comp	-	Comp	-
	Q	Comp	-	Comp	-
Steady-State Random Bi Bi	P	Mixed	Mixed	Mixed	Mixed
	Q	Mixed	Mixed	Mixed	Mixed

Unsat - initial rates measured at a constant unsaturating concentration of substrate
Sat - initial rates measured at a constant saturating concentration of substrate
Comp - Competitive inhibition
Uncomp - Uncompetitive inhibition
Mixed - Mixed inhibition

REFERENCES

1. Cleland, W. W. (1963) *Biochimica et Biophysica Acta* **67**, 104-137
2. Segel, I. H. (1975) *Enzyme Kinetics: Behaviour and Analysis of Rapid Equilibrium adn Steady-State Enzyme Systems*, John Wiley & Sons, Inc., New York
3. Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A., Lopez, R., Thompson, J. D., Gibson, T. J., and Higgins, D. G. (2007) *Bioinformatics* **23**, 2947-2948