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Anomeric Specificity of L-Fucose Dehydrogenase: A Stereochemical Imperative in Aldopyranose Dehydrogenases?†

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ABSTRACT: A set of hypotheses is proposed that explains the anomeric specificity of aldopyranose dehydrogenases in terms of an evolutionarily selected function. The first hypothesis, based on stereoelectronic theory, argues that, in the "allowed" transition state for oxidation at the anomeric carbon, the two oxygens attached to the anomeric carbon each bear a lone pair of electrons antiperiplanar to the departing "hydride". The second hypothesis is that the dehydrogenase is functionally constrained to bind the anomer that has this arrangement of lone pairs in its lowest energy chair conformer. The anomeric specificity of L-fucose dehydrogenase is experimentally examined. The enzyme oxidizes preferentially the β-anomer, consistent with the prediction made by these hypotheses. Available experimental data for other enzymes (D-glucose-6-phosphate dehydrogenase, D-glucose dehydrogenase, D-galactose dehydrogenase, D-abequose dehydrogenase, and D-arabinose dehydrogenase) are found to be also consistent with the proposed hypotheses.

For more than a quarter of a century, it has been known that certain aldose dehydrogenases act on only one anomer of their sugar substrates. In 1952, Strecker and Korkes (Strecker & Korkes, 1952) showed that an NAD^+-dependent[1] D-glucose dehydrogenase selectively catalyzes the oxidation of the β-anomer,[2] and a half-dozen other enzymes have been examined

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[1] Abbreviations: NAD^+, nicotinamide adenine dinucleotide, oxidized form; NADH, nicotinamide adenine dinucleotide, reduced form; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

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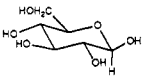
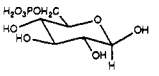
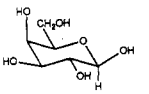
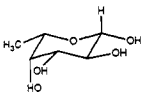
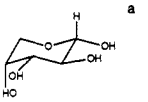
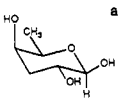
Table I: Experimental Anomeric Specificity of Aldose Dehydrogenases Dependent on Nicotinamide Cofactors Compared with the Specificity Expected on the Basis of Hypotheses Presented in Text

ENZYME	SOURCE	PRESUMED REACTIVE CONFORMATION	ANOMERIC SPECIFICITY		ABSOLUTE ANOMERIC CONFIGURATION	REF.
			Expected	Found		
D-Glucose Dehydrogenase E.C.1.1.1.47	Ox liver		β	β	R	c
D-Glucose-6-Phosphate Dehydrogenase E.C.1.1.1.49	Yeast		β	β	R	d
D-Galactose Dehydrogenase E.C.1.1.1.48	*Pseudomonas saccharophila* *Pseudomonas fluorescens*		β	β	R	e
L-Fucose Dehydrogenase E.C.1.1.1.122	Pig liver		β	β	S	This paper
D-Arabinose Dehydrogenase E.C.1.1.1.117	*Pseudomonad*	 [a]	α	α	S	f
D-Abequose Dehydrogenase	*Pseudomonas putida*	 [a]	β	β [b]	R	g

[a] Initial reaction product not rigorously proven. [b] Determined only with D-galactose. [c] Strecker & Korkes, 1952. [d] Salas et al., 1965. [e] Wallenfels & Kurz, 1962; Ueberschar et al., 1974. [f] Cline & Hu, 1965. [g] Schiwara & Domagk, 1968.

since (Salas et al., 1965; Wallenfels & Kurz, 1962; Ueberschar et al., 1974; Schiwara & Domagk, 1968; Cline & Hu, 1965). However, there has not yet emerged a rationalization for the particular anomeric preferences of specific enzymes. In fact, in 1976, Benkovic commented that "the observed β-anomeric specificity [of D-glucose-6-phosphate dehydrogenase] is not readily rationalized at present in terms of a mechanism for the redox ... reaction. It would appear that either anomer should have sufficed" (Benkovic & Schray, 1976).

Members of classes of enzymes catalyzing analogous reactions generally display common stereoselectivities (Hanson & Rose, 1975). We have been concerned with determining whether classes of enzymes that violate this generalization do so for functional reasons or whether the divergent behavior is nonfunctional, reflecting pedigree or randomness (Benner, 1982; Nambiar et al., 1983; Rozzell & Benner, 1984). This issue is important, as it helps distinguish those behaviors of enzymes that are the products of natural selection, and therefore suited to functional interpretation, from those that are not. Thus, examining this question can further define the paths of protein evolution.

Hypotheses directed to this point are not readily "proven". Therefore, the strategy must be to search for a functional theory that makes predictions regarding the stereoselectivity of relevant enzymes and then to test these predictions by examining enzymes that have not yet been studied. At this point, there is no theory for predicting the stereoselectivity of aldose dehydrogenases that is "conventional wisdom" (vide supra). Therefore, a working hypothesis is needed to stimulate and guide future work.

We offer here such a hypothesis, based on the theory of "stereoelectronic control" (Deslongchamps, 1983), that rationalizes the anomeric preferences of aldopyranose dehydrogenases dependent on nicotinamide cofactors (Table I).[3] Further, we wish to report that L-fucose dehydrogenase (EC 1.1.1.122) acts on the β-anomer having the *S* absolute configuration at carbon 1, consistent with the predictions of this hypothesis.

Hypotheses

Stereoelectronic theory makes explicit statements regarding the removal of a "hydride ion" from carbon 1 of an aldopyranose. It is possible for a hydrogen in the axial position to be antiperiplanar to two lone pairs of electrons in the chair conformation. A hydrogen in the equatorial position can be antiperiplanar to at most one lone pair. Thus, we define a "stereoelectronically allowed geometry" in this case as one where two lone pairs can donate into the σ^*-orbital of the C–H bond of the departing hydride. The donation of two pairs of electrons permitted by the allowed geometry lowers the energy of the "allowed" transition state relative to alternative transition states with different geometries, where only one lone pair is

[2] Anomers are named in accordance with the conventions described by Kennedy and White (1983); note that the convention in sugar chemistry is different from that in steroid chemistry and denotes the relative stereochemistry of the anomeric center and the asymmetric center farthest from the aldehyde.

[3] Although the arguments use the formalism of stereoelectronic theory, alternative arguments based on "least nuclear motion" make similar predictions (Sinnott, 1984; Hosie et al., 1984). Sinnott's critique of stereoelectronic theory as applicable to acetal hydrolysis does not necessarily challenge our interpretation here. The transition state for these oxidation reactions, where the incipient carbonium ion is stabilized by two lone pairs, is earlier than the "late" transition states found in acetal hydrolysis, where only one pair stabilizes the incipient carbonium ion.

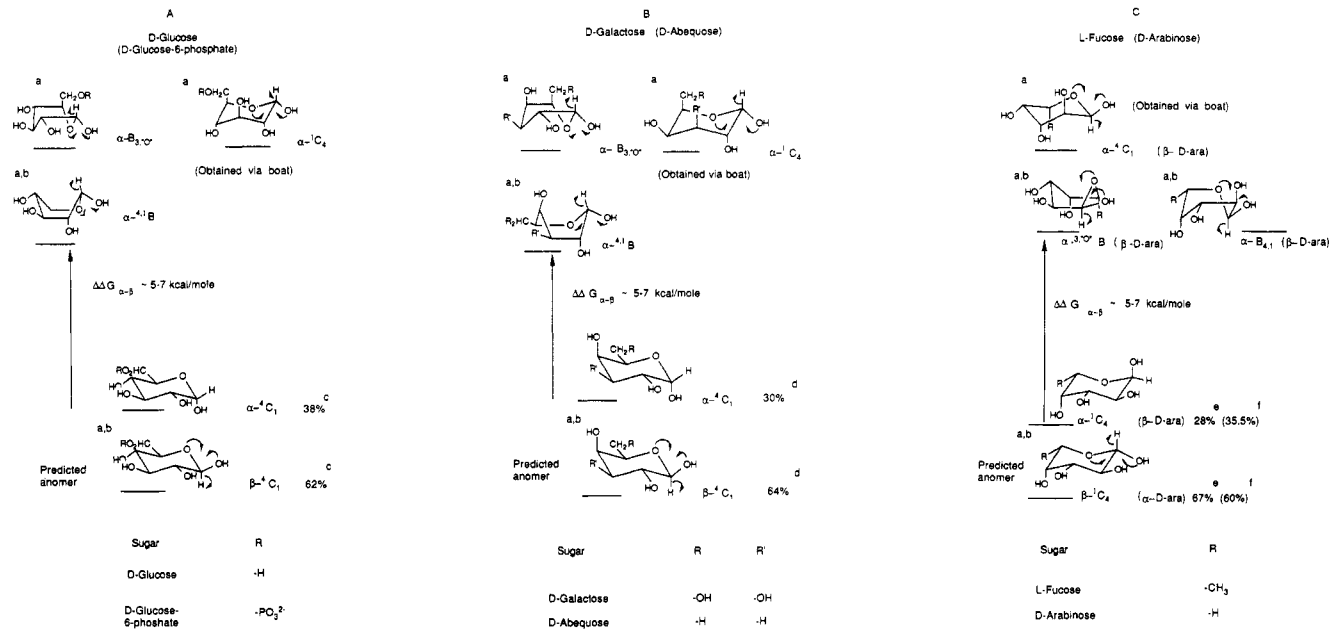


FIGURE 1: Energy diagram showing approximate relative energies of the ground-state conformations of both anomers of representative sugars. For any particular sugar, only one of the anomers has two lone pairs of electrons antiperiplanar to the departing hydride in its lowest energy conformer. This is the conformer that can undergo stereoelectronically allowed hydride transfer. For the anomer that does not have the stereoelectronically desired disposition of lone pairs in its lowest energy conformer, the energy diagram also shows higher energy conformations that permit stereoelectronically allowed transfer of hydride. Predictions of anomeric specificity are made by presuming that dehydrogenases have evolved to catalyze hydride transfer from the lowest energy conformation that permits departure of hydride according to stereoelectronic rules. (a) Denotes a conformer that permits stereoelectronically allowed hydride transfer. (b) Denotes the lowest energy conformation that permits stereoelectronically allowed hydride transfer for each anomer. (c) Angyal, 1984. (d) For D-galactose. (e) For L-fucose (5% furanose). (f) For D-arabinose.

available to stabilize the incipient carbonium ion.

Thus, if aldopyranose dehydrogenases have evolved to catalyze reactions along the stereoelectronically allowed pathway, they will transfer an axial hydrogen. If we introduce an additional assumption that these enzymes act on the lowest energy chair conformation of their substrate, their anomeric preference will be for the anomer that has the C-1 hydrogen axial in the lowest energy conformer.

These ideas can be formulated as a set of hypotheses that makes experimentally testable predictions: (1) The most efficient catalysis of the expulsion of hydride requires that the transition state have two oxygen lone pairs oriented antiperiplanar to the departing hydride. This is the conformation that can transfer hydride via a stereoelectronically allowed pathway (Deslongchamps, 1983). (2) The optimal[4] mode of obtaining two antiperiplanar lone pairs is by binding the anomer that provides them in the ground conformational state, as opposed to binding a high-energy conformation of an anomer that does not provide the requisite lone pairs in the ground conformational state (Figure 1). (3) Aldopyranose dehydrogenases have evolved to be optimally efficient catalysts.

These hypotheses predict the anomeric specificity of aldose dehydrogenases that satisfy the following conditions: (1) A strong argument can be made regarding the evolutionarily relevant "natural substrate(s)" of the enzyme. (2) The cofactor is a pyridine nucleotide. (3) The initial product of the reaction is the aldono-1,5-lactone.

The first restriction is necessary because natural selection is the only mechanism for obtaining functional behavior in living systems. Therefore, a functional theory based on the properties of the natural substrate can be predictive only to the extent that the evolutionarily relevant natural substrate is defined. The second restriction presumably constrains us to examine enzymes acting through hydride-transfer mechanisms. The third restriction excludes simple aldehyde oxidation, where the α- and β-anomers of the substrate are not relevant to the microscopic reaction mechanism. Further, it constrains us to examine enzymes acting on pyranoses. Although the oxidation of furanoses should not violate stereoelectronic hypotheses, the flexibility of furanoses makes it difficult to analyze their conformational energetics.

These hypotheses account for the observed stereospecificity of all aldopyranose dehydrogenases examined so far (Table I). Further, they predict that enzymes oxidizing L-fucose (6-deoxy-L-galactose) to L-fucono-1,5-lactone will act on the β-anomer, as it is this anomer of L-fucose that has a ground-state conformer with the requisite lone pairs antiperiplanar to the leaving hydride (Figure 1).

Fucose dehydrogenase from pig is a well-characterized aldopyranose dehydrogenase with unknown anomeric specificity.[5] Further, this enzyme acts on an L sugar with a $^1C_4$

[4] "Optimal" is understood in the context of Darwinian natural selection, where organisms possessing less efficient enzymes are less able to survive and reproduce. In the context of these stereoelectronic proposals, catalysis of a dehydrogenation reaction on the "favored" anomer is intrinsically more facile than catalysis of this reaction on the "unfavored" anomer. By this argument, the selective advantage of enzymes selecting the "favored" anomer is derived from the presumption that the amount of these enzymes needed to catalyze the physiologically desired flux can be made at a lesser metabolic cost.

[5] L-Fucose (6-deoxy-L-galactose) is a naturally occurring monosaccharide found in numerous glycoproteins and glycolipids (Ginsburg & Neufeld, 1969), including blood group A substance from hog gastric mucin (Bray et al., 1946). GDP-L-fucose serves as a fucosyl donor in the biosynthesis of complex carbohydrates (Foster & Ginsburg, 1961). A β-galactoside α-(1→2)-fucosyltransferase has been isolated from porcine submaxillary gland (Sadler et al., 1982). GDP-L-fucose is synthesized from D-glucose via GDP-D-mannose in bacteria, plants, and pigs (Ginsburg, 1961; Liao & Barber, 1971; Ishihara & Heath, 1968). L-Fucose dehydrogenase catalyzes the NAD^+-dependent oxidation of L-fucose to L-fucono-1,5-lactone in mammals (Schachter et al., 1969). The catabolic pathway in porcine liver that converts L-fucose to two molecules of L-lactate has been elucidated (Chan et al., 1979).

ground-state conformation,[6] in contrast with the D-hexoses, which have a distinct $^4C_1$ ground-state conformation. The absolute stereochemistry at carbon 1 is the opposite of that for the other hexoses whose dehydrogenases have been examined. Therefore, the anomeric specificity of the enzyme is less likely to be explainable in terms of a simple argument based on pedigree.

Experimental Procedures

Materials. NAD^+, α-L-fucose, and porcine liver L-fucose dehydrogenase (1 unit/mg) were purchased from Sigma. One unit of enzyme will oxidize 1 μmol of L-fucose/min to L-fucono-1,5-lactone at pH 8.0 and 37 °C. On native isoelectric focusing, the enzyme appears by staining with Coomassie blue as one major band with a p*I* of 5.0 with two minor bands having slightly lower p*I* (4.9–5.0). By activity staining with L-fucose, NAD^+, phenazine methosulfate, and nitroblue tetrazolium, all three bands are catalytically active. SDS–polyacrylamide gel electrophoresis suggests a monomeric molecular weight of 30000. The enzyme has a specific activity comparable to that reported for pure enzyme (Schachter et al., 1969; Chan et al., 1979).

Studies of Mutarotation. A solution of α-L-fucose (9.2 mg/mL, 25 mM Tris, pH 7.0) was prepared and immediately introduced into a polarimeter cell. Optical rotation was followed with a Perkin-Elmer Model 241 polarimeter (Na D line, 25 °C), and data were fitted to kinetic equations describing the approach to equilibrium.

Anomeric Specificity. A solution of fucose dehydrogenase was dialyzed against several changes of buffer (25 mM Tris or 25 mM potassium phosphate, pH 7.0, 20 units/mL) to remove contaminating L-fucose. To solutions of the enzyme (6 units/mL) in buffer with NAD^+ (0.50 mM) was added a freshly prepared solution of L-fucose (46 μM) (Figure 2a), consisting primarily (95%) of the α-anomer. In parallel experiments (Figure 2b), a solution of fucose dehydrogenase was added to a solution of fucose that had been preincubated for 113 min (ca. 10 mutarotation half-times) to give an equilibrium mixture of the β- and α-anomers of the pyranose form, with some furanose, in the approximate ratio of 67:28:5 (Angyal, 1984).

Results

The half-time for the mutarotation of α-L-fucose is approximately 11.5 min at pH 7. This is slow enough to permit direct measurement of the anomeric specificity of the dehydrogenase. Figure 2 shows the results of incubation of excess dehydrogenase with α-L-fucose and an equilibrium mixture of α- and β-L-fucose. With the equilibrium mixture (Figure 2b), NADH corresponding to 65 ± 6% of total fucose[7] is formed in a rapid first step, followed by a slower conversion of the remaining fucose at a rate corresponding to the mutarotation rate. There is no rapid first step when essentially pure α-anomer (Figure 2a) is presented to the dehydrogenase. The total added fucose is oxidized at a rate characteristic of the mutarotation rate.

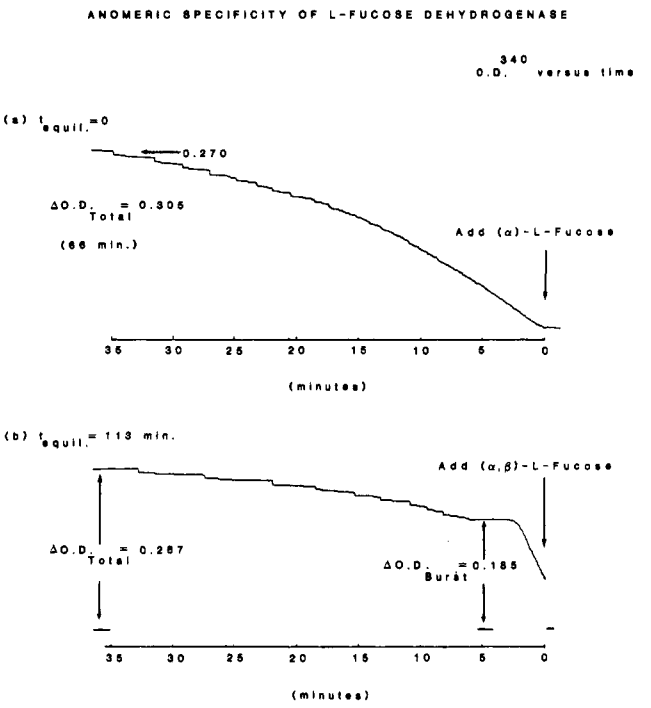


FIGURE 2: Optical density (OD) at 340 nm as a function of time for oxidation by L-fucose dehydrogenase of equivalent amounts of (a) α-L-fucopyranose and (b) ca. 67% β-L-fucopyranose, 27% α-L-fucopyranose, and 5% L-fucofuranose. Reaction mixture contains 25 mM Tris, 500 μM NAD^+, 46 μM L-fucose, and 6 units of fucose dehydrogenase (pH 7, 25 °C). Note that in part b, after 113 min, approximately 10 half-lives for mutarotation have elapsed. The burst observed in part b is not present in part a, suggesting that fucose dehydrogenase acts on the β-anomer.

We interpret these kinetic plots as evidence for a rapid initial oxidation of the β-pyranose form of L-fucose, which comprises 67% of the equilibrium mixture (Angyal, 1984). The remaining 33% L-fucose is then oxidized at a slower rate, with mutarotation rate limiting under the conditions of the assay. Thus L-fucose dehydrogenase is specific for the β-anomer of L-fucopyranose, consistent with the hypotheses proposed above. Further, the absolute stereochemistry at carbon 1 for the preferred anomer of L-fucose is *S*, opposite that for the preferred anomers of the other hexose dehydrogenases.

Discussion

A decade ago, Hanson and Rose suggested that similar stereochemical preferences exhibited by enzymes catalyzing related reactions might reflect a "mechanistic imperative" (Hanson & Rose, 1975). Similar proposals have since been made for many classes of enzymes, including dehydrogenases (Benner, 1982; Nambiar et al., 1983), decarboxylases (Rozzell & Benner, 1984), and phosphoryl transferases (Sheu et al., 1979).

While aldopyranose dehydrogenases are expected to select one anomer over the other (Wurster & Hess, 1974), this expectation is not an explanation for why any particular anomer is chosen. Several alternative functional explanations are possible. For example, it might be that enzymes evolve to act on the anomer that predominates at equilibrium (Benkovic & Schray, 1976). While this explanation is consistent with data on aldose dehydrogenases in Table I, it is not consistent with data for several aldose kinases and aldose isomerases (Howard & Heinrich, 1965; Heinrich & Howard, 1966; Feather et al., 1970; Cori et al., 1937; Rose et al., 1973). Further, in several cases, since the equilibrium populations of the two anomers are nearly identical, it seems unlikely that

[6] Standard nomenclature is used to describe the conformations of sugars. A reference plane is selected that includes four ring atoms, and the exoplanar atom projecting through the side of the plane from which the ring numbering appears clockwise is given a superscript. The atom projecting from the plane on the opposite side is given a subscript (Ferrier & Collins, 1972; Stoddart, 1971).

[7] Based upon an average total optical density change for the two experiments shown.

the survival of the host depends on selecting the anomer with 60% abundance at equilibrium. Therefore, we consider this argument less reasonable as a working hypothesis.

Our functional explanation for the anomeric preferences of aldopyranose dehydrogenases is based on two hypotheses: that, in these cases, evolution selected for enzymes that conform to stereoelectronic principles and for enzymes that use as substrate the ground-state conformer. Both hypotheses are important, and the argument presumes that natural selection prefers enzymes that obtain the appropriate arrangement of lone pairs by one geometric approach (binding one of two anomers) over an alternative approach that presumably "costs" more (binding a high-energy conformation of an alternative anomer).[4]

Either of these assumptions might be regarded as controversial. For example, although stereoelectronic principles (Deslongchamps, 1983) are widely used to analyze the stereochemical course of enzymatic reactions, including lysozymes (Chipman & Sharon, 1969; Ford et al., 1974,; Gorenstein et al., 1977), proteases (Bizzozero & Zweifel, 1975; Bizzozero & Dutler, 1981), dehydrogenases (Benner, 1982; Nambiar et al., 1983), and phosphatases (Taira et al., 1984), their use has not been unchallenged, both at the level of data (Kluger & Thatcher, 1985) and at the level of theory.[3]

Historical explanations that make presumptions about the origin of enzymes and constraints governing their evolution are also possible (Benner et al., 1985). For example, enzymes that obey stereoelectronic rules may simply be "kinetically" easier to evolve than those that do not. Further, enzymes that obtain the appropriate arrangement of lone pairs in the active site by binding the "correct" anomer may simply be easier to evolve than those that bind a high-energy conformer of the "wrong" anomer. The increased complexity of such models does not make them less likely to be true, but it does make them more difficult to test.

The most reasonable historical model (Benner et al., 1985) divides the enzymes in Table I into two classes, each representing a separate line of descent. L-Fucose dehydrogenase and D-arabinose dehydrogenase are in one class, and the remaining dehydrogenases are in the other. Anomeric specificity is the result of separate historical accidents in the two ancestral dehydrogenases. Once established, anomeric specificity is highly conserved. Thus, the model assumes that the identical preference for presumed axial hydride transfer in the two classes is coincidental (i.e., not functional).

This historical model has several weaknesses. First, it is somewhat ad hoc.[8] Any collection of data can be explained historically by postulating a sufficient number of historical accidents and independent pedigrees. Further, the explanation presumes that there are nonfunctional constraints on the "drift" of anomeric specificity that are more stringent than constraints on the "drift" of substrate specificity. Therefore, we believe that the functional model is best suited for experimental test.

We report here the results of the first predictive test of a *functional* theory explaining the anomeric specificity of aldopyranose dehydrogenases. More experiments are clearly necessary to provide a full understanding of anomeric specificity in enzymology.[9] We hope this work encourages these experiments.

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Registry No. EC 1.1.1.122, 9082-70-6; EC 1.1.1.47, 9028-53-9; EC 1.1.1.49, 9001-40-5; EC 1.1.1.48, 9028-54-0; EC 1.1.1.117, 37250-47-8; D-abequose dehydrogenase, 107269-56-7; aldopyranose dehydrogenase, 107269-57-8.

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[8] Sequence data may not provide conclusive evidence concerning the relatedness of enzymes that are widely divergent. Evidence for or against relatedness in these cases might only be obtainable from crystal structures.

[9] One referee has made the point that these hypotheses do not suggest a mechanism by which the anomeric specificity might have evolved, as the stereoelectronic argument based on the orientation of lone pairs in the aldopyranose substrate naively appears to not be relevant to an enzyme that has evolved to reduce the lactone by directing an attack of a hydride to one or the other face of the lactone. In fact, by microscopic reversibility, the stereoelectronic argument must apply to both the forward (oxidative) and reverse (reductive) reactions. In the reductive direction, the lone pair on the ring oxygen is expected to be reoriented antiperiplanar to the incoming hydride. For "α" attack of L-fuconolactone, this stereoelectronic consideration would create either a boat conformer or a high-energy chair conformer. For "β" attack, the ground-state chair conformer would be expected as the product.

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Slow-Binding Inhibition of Chymotrypsin and Cathepsin G by the Peptide Aldehyde Chymostatin

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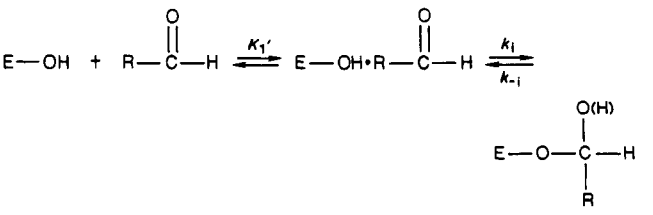
ABSTRACT: The microbial, peptide-derived aldehyde chymostatin is a potent, competitive inhibitor of chymotrypsin and cathepsin G: $K_i = 4 \times 10^{-10}$ and 1.5×10^{-7} M, respectively. It is a "slow-binding inhibitor" of both proteases and, as such, allows determination of rate constants for its association with and dissociation from these proteases. Inhibition kinetics indicate second-order rate constants for the association of chymostatin with chymotrypsin and cathepsin G of 360 000 and 2000 M^{-1} s^{-1}, respectively, and a first-order rate constant for the dissociation of both protease–chymostatin complexes of approximately 0.0002 s^{-1}. Thus, the extreme difference in potency of chymostatin as an inhibitor of chymotrypsin and cathepsin G originates entirely in k_{on}. Solvent deuterium isotope effects (SIE) were determined to probe the reaction step that rate limits k_{on}. For the reaction of chymotrypsin with chymostatin, the SIE for k_{on} is 1.6 ± 0.1, while for the reaction of chymotrypsin with the peptide substrates Ala-Ala-Phe-pNA and Suc-Ala-Ala-Pro-Phe-pNA, the SIE's for k_c/K_m are 2.8 ± 0.2 and 1.9 ± 0.1, respectively. These results suggest that k_{on} for the association of chymotrypsin with chymostatin is at least partially rate limited by a reaction step involving proton transfer. Combined with results for the inhibition of chymotrypsin by Bz-Phe-H [Kennedy, W. P., & Schultz, R. M. (1979) *Biochemistry 18*, 349–356], these data suggest a mechanism for inhibition by chymostatin involving the general-base-catalyzed formation of an enzyme-bound hemiacetal, followed by a conformational change of this intermediate that produces the final, stable complex of enzyme and inhibitor. For the inhibition of chymotrypsin, the transition states of these two reaction steps are of similar energy, and each partially rate limits the overall process governed by k_{on}.

Chymostatin is a microbial inhibitor of serine proteases (Umezawa et al., 1970; Tatsuta et al., 1973; Umezawa, 1976; Feinstein et al., 1976; Marossy et al., 1981; Bromme & Kleine, 1984; Galpin et al., 1984). It is isolated from various strains of *Streptomyces* as three forms, chymostatins A, B, and C, that differ only in the amino acid residue at the second position (Chart I). The major form is chymostatin A and typically accounts for greater than 80% of the mixture (Delbaere & Brayer, 1985). The Phe residue at the primary position predisposes the inhibitor for binding to chymotrypsin and other serine proteases having a primary specificity for Phe.

Scheme I



Chymostatin, like other peptide-derived aldehydes, is thought to inhibit serine proteases according to the minimal

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