Structure-Specificity Relationship of Cardiac Glycosides as a Substrate for Glucohydrolase II

Yukimasa TERADA,*[,a] Rumi MISOI,[a] Naoharu WATANABE,[b] Martin HORNBERGER,[c] and Wolfgang KREIS[c]

Faculty of Pharmacy, Meijo University,[a] Yagotoyama, Tenpaku, Nagoya 468–8503, Japan, Department of Applied Biological Chemistry, Shizuoka University,[b] Ohya, Shizuoka 422–8529, Japan, and Lehrstuhl für Pharmazeutische Biologie, Friedrich-Alexander-Universität,[c] Staudstraße 5, 91058 Erlangen, Germany.

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Cardenolide glucohydrolase II (CGH II) is a cardenolide-specific glucohydrolase obtained from *Digitalis lanata* leaves. We investigated the structure–specificity relationship of several cardenolide disaccharides as a substrate for CGH II. Conformation analysis of the substrates was performed using molecular mechanics calculations. The sugar chain conformation of two inert glycosides was significantly different from that of the other glycosides. The other two glycosides, which were weak substrates of CGH II, were suggested to have an intramolecular hydrogen bond between the sugar groups. It was deduced that this hydrogen bond restricts the conformational change of the sugar chain and prevents the glycosides from enzymatic recognition.

Key words cardenolide glucohydrolase II (CGH II); cardiac glycoside; structure–specificity relationship; conformation analysis; molecular mechanics

The leaves of *Digitalis lanata* contain large amounts of cardiac glycosides, most of which belong to a group termed primary glycosides. The common feature of these glycosides is a terminal glucose residue at the end of the sugar side chain.[1)] During the isolation of primary cardiac glycosides, enzymatic hydrolysis may occur that leads to the so-called secondary glycosides.

Stoll *et al.*[2)] reported the enzyme activities in *Digitalis* leaves capable of hydrolyzing primary glycosides to their corresponding secondary glycosides. Since then, two cardenolide-specific glucohydrolases termed cardenolide glucohydrolase I (CGH I) and cardenolide glucohydrolase II (CGH II) have been isolated from various *Digitalis* species. CGH I characteristically hydrolyzes cardenolide tetrasaccharides,[3)] whereas CGH II is specific for the cardenolide disaccharides.[4)] Glucoevatromonoside (**1**) is the main substrate of CGH II and the velocity of the deglucosylation decreases by the substitution of the inner sugar (digitoxose) with glucose (**2**), glucomethylose (**3**), fucose (**5**), and digitalose (**6**) (for structural formulas, see Fig. 1 and Table 1).

In this paper, the substrate selectivity of CGH II is discussed in connection with the sugar chain conformation of the cardiac glycosides. The Search-Compare routine of the Insight II program package[5)] was used to scan all possible conformations of the genin–sugar and sugar–sugar linkages. It was inferred that an intramolecular hydrogen bond restricts the rotation of the sugar–sugar linkage and this may hinder the conformational change relevant to the enzymatic recognition.

Experimental

The cardenolide disaccharides are hydrolyzed as substrates for CGH II releasing the terminal glucose. The molecular structures of the substrates studied in this work are shown in Fig. 1 and Table 1. The relative velocity of the deglucosylation is shown in Table 1, and the detailed kinetic constants of the catalytic deglucosylation by CGH II will be reported by Hornberger and Kreis[4)].

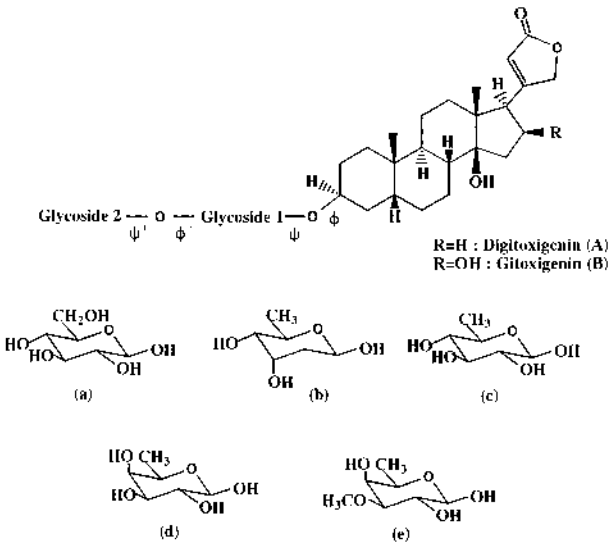

Fig. 1. Chemical Structures of the Cardenolide Genins and Sugars Occurring in the Cardiac Glycosides in the Text

(a) β-D-glucose, (b) β-D-digitoxose, (c) β-D-glucomethylose, (d) β-D-fucose and (e) β-D-digitalose.

Table 1. Structure and Deglucosylation Velocity of the Cardiac Glycosides

Compound	Genin[a)]	Glycoside 1	Glycoside 2	Velocity at 1 mM substrate (%)[b)]
Glucoevatromonoside (**1**)	A	β-D-digitoxose	β-D-glucose	100
Cellobioside (**2**)	A	β-D-glucose	β-D-glucose	52
Glucodigimethyloside (**3**)	A	β-D-glucomethylose	β-D-glucose	38
Glucogitroside (**4**)	B	β-D-digitoxose	β-D-glucose	33
Glucodigifucoside (**5**)	A	β-D-fucose	β-D-glucose	0
Glucodigitaloside (**6**)	A	β-D-digitalose	β-D-glucose	0

a) See Fig. 1. *b*) Substrate preferences of CGH II (see ref. 4).

* To whom correspondence should be addressed.

Table 2. The Calculated Energy and the Geometry of the Conformations within 1.8 kcal/mol of the Global Minimum

Compound		Relative energy (kcal mol^{-1})	Dihedral angle (°)[a]				H···O Distance[b] (Å)
			ϕ	Ψ	ϕ'	Ψ'	
Glucoevatromonoside (**1**)	1	0.00	−89	172	103	170	3.40
	2	0.00	−89	172	102	170	3.37
	3	0.00	−89	172	75	153	2.42
	4	0.05	−89	172	101	169	3.32
	5	0.44	−131	177	103	170	3.41
	6	0.44	−131	177	103	170	3.39
	7	0.44	−131	177	103	170	3.38
	8	0.44	−131	177	75	153	2.42
	9	0.44	−124	176	103	170	3.41
	10	0.80	−89	172	88	98	3.19
	11	0.91	−145	−169	123	179	4.03
	12	1.24	−131	177	88	98	3.19
	13	1.24	−130	177	88	98	3.19
	14	1.24	−132	177	89	97	3.20
	15	1.25	−145	−180	118	−77	4.58
	16	1.26	−130	176	88	100	3.15
	17	1.58	−89	172	114	−74	4.52
Cellobioside (**2**)	1	0.00	150	169	103	164	1.72
	2	0.93	150	169	103	164	1.72
	3	1.40	109	174	103	164	1.72
	4	1.40	108	174	103	164	1.72
	5	1.40	110	174	103	164	1.72
	6	1.40	112	174	103	164	1.72
Glucodigimethyloside (**3**)	1	0.00	−89	169	107	169	1.73
	2	0.49	−127	174	107	169	1.73
	3	0.49	−128	174	107	169	1.73
	4	0.49	−128	174	107	169	1.73
	5	0.49	−129	174	107	169	1.73
	6	0.49	−130	174	107	169	1.73
	7	1.01	−145	−170	112	178	1.73
	8	1.02	−150	95	107	169	1.78
	9	1.18	−104	−80	107	169	1.73
	10	1.54	−162	−115	107	170	1.73
Glucogitroside (**4**)	1	0.00	−89	172	103	170	3.41
	2	0.00	−89	172	103	170	3.40
	3	0.00	−89	172	102	169	3.36
	4	0.01	−89	172	75	153	2.43
	5	0.01	−89	172	75	153	2.41
	6	0.03	−89	172	105	172	3.47
	7	0.43	−131	177	103	170	3.40
	8	0.44	−131	177	75	153	2.42
	9	0.80	−89	172	88	98	3.19
	10	0.94	−145	−170	123	179	4.03
	11	1.23	−131	177	88	98	3.19
	12	1.29	−145	−180	118	−77	4.58
	13	1.58	−89	172	114	−74	4.51
Glucodigifucoside (**5**)	1	0.00	−141	−178	−99	135	3.99
	2	0.08	−139	−179	61	155	1.87
	3	0.08	−139	−179	61	155	1.87
	4	0.09	−139	−178	61	155	1.87
	5	0.14	−91	170	61	155	1.84
	6	0.58	−109	81	68	156	2.03
	7	0.99	−164	−111	−95	134	3.96
	8	1.02	−109	74	63	155	1.92
	9	1.18	−89	169	−97	137	3.95
	10	1.24	−147	94	62	155	1.85
	11	1.33	−118	−76	64	156	1.90
	12	1.67	−110	81	68	156	2.03
Glucodigitaloside (**6**)	1	0.00	−139	−179	−112	134	—
	2	0.03	−144	−174	−101	170	—
	3	0.07	−165	−109	−92	175	—
	4	0.41	−144	−176	−107	168	—
	5	0.77	−89	169	−96	175	—
	6	0.77	−166	−112	−90	175	—
	7	0.97	−132	177	−140	87	—
	8	1.10	−134	177	−95	175	—
	9	1.15	−88	168	−97	176	—
	10	1.62	−88	169	−140	87	—
	11	1.64	−113	−78	−143	89	—
	12	1.71	−117	−77	−143	89	—

a) ϕ: C4-C3-O-C1′; Ψ: C3-O-C1′-C2′; ϕ': C3′-C4′-O-C1″; Ψ': C4′-O-C1″-C2″. *b*) Non-bonding distance between the C3′-OH and the glucopyranosyl oxygen. Hydrogen bond is plausible to the underlined distance.

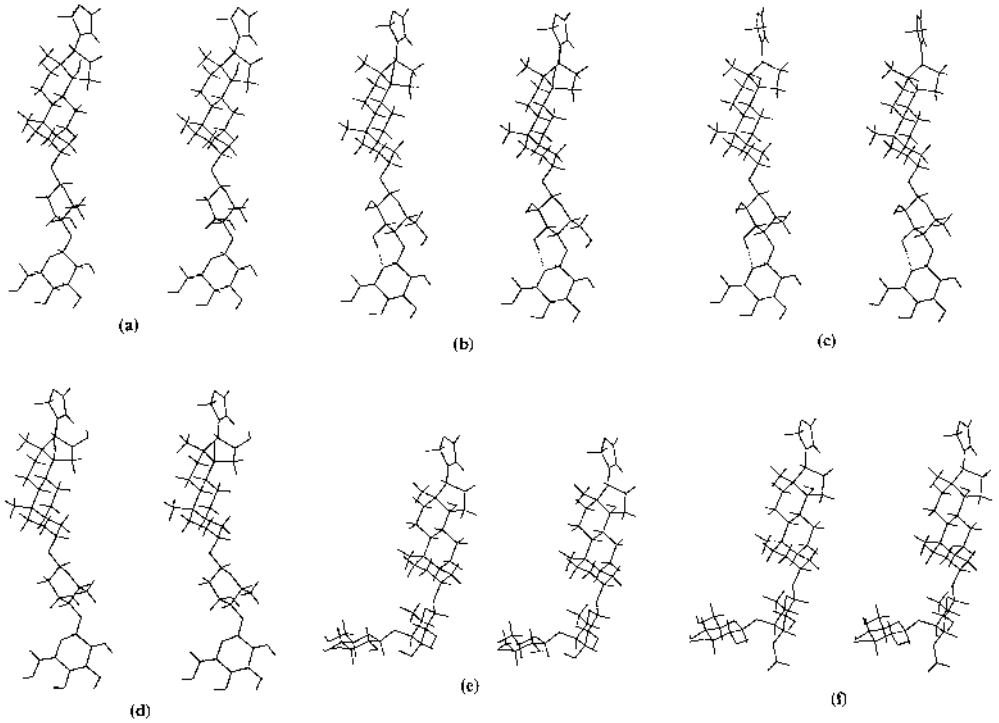

Fig. 2. Stereoview of the Most Stable Conformation of the Cardiac Glycosides

A broken line shows the possible hydrogen bond. (a) glucoevatromonoside (**1**), (b) cellobioside (**2**), (c) glucodigimethyloside (**3**), (d) glucogitoroside (**4**), (e) glucodigifucoside (**5**) and (f) glucodigitaloside (**6**).

The conformational analysis of the individual substrate was performed by using Insight II[5] and Discover[6] modeling and simulation softwares on an O2 workstation (OS: IRIX 5.2) from Silicon Graphics.

Starting coordinates were generated by using the drawing tool of Insight II,[5] and geometry optimization was carried out using the CFF91 force field of the Discover program.[6] All calculations were performed in a vacuum. In the CFF91 force field, hydrogen bonds are a natural consequence of the standard van der Waals and electrostatic parameters, thus hydrogen bond functions were not used in particular. The dielectric constant (relative permittivity) was 1.00 and the cutoff distance was 9.5 Å. The optimization was terminated when average derivatives reached less than 0.01 kcal/mol·Å or at 5000 iterations.

The three cyclohexane rings of the digitoxigenin framework were assumed to be in the chair form as reported by Allinger and Wu.[7] The orientation of the butenolide ring was not specified during the entire calculation process. The intramolecular flexibility was then completely optimized using the CFF91 force field of the Discover program. Subsequently, the four bonds, namely C3-O (ϕ), O-C1′ (Ψ), C4′-O (ϕ'), and O-C1″ (Ψ') (Fig. 1), were rotated in increments of 60° using the Search-Compare routine. Among the numerous conformations thus generated, the conformers that had atoms within the van der Waals contact zone were automatically eliminated. In this elimination step, the van der Waals radius of all atoms was reduced by 70% of the default value in order to get sufficient conformations. Starting from these conformations, structural optimization by the CFF91 force field was again performed. During this geometry optimization, all internal coordinates including the four dihedral angles were relaxed.

Results

The four C–O bonds, which define the overall shape of the molecule, were rotated in increments of 60° and the conformers without the van der Waals contact were selected using the Search-Compare routine. After the energy minimization of these conformers, each cardiac glycoside resulted in dozens of stable conformations. However, the results include a number of similar conformations with comparable total energy and similar dihedral angles. This means that there are only a few low barriers on the potential energy hypersurface. Therefore, even when the starting conformers have a dihedral difference of more than 60°, they can converge to the same conformation overcoming energy barriers in the minimization process. In Table 2, we have listed the low energy conformations within 1.8 kcal/mol of the global minimum, since a 1.8 kcal energy difference indicates that 95% of the conformers exist in the stable form. In this table, the four dihedral angles (ϕ, Ψ, ϕ', and Ψ') and the nonbonded distance between the hydrogen atom of C3′-OH of the inner sugar and the oxygen atom of the terminal glucopyranose ring are also shown. A hydrogen bond was assumed when the H···O distance was shorter than 1.8 Å, because the distance between the electronegative atoms in the hydrogen bond is usually in the range of 2.5—2.7 Å.[8] The appearance of the most stable conformation of each compound is shown in Fig. 2.

Discussion

The overall conformation of cardiac glycosides is defined by three fragments having the ability to rotate around the four C–O bonds. One of these fragments is the digitoxigenin group that is rigid except for the rotation of the butenolide ring. The others are the sugar rings, which take the chair conformation. The mutual positions of the genin and the saccharide fragments play a crucial role with respect to the overall shape of the cardiac glycosides. When a cardenolide disaccharide reacts under the catalytic control of CGH II, the genin and the two saccharide fragments must fit into the active site of the enzyme. As glucoevatromonoside (**1**) is the best substrate for CGH II, this molecule can take the best position and orientation in the enzyme's active site. Contrarily, there must be some reason that the other less reactive compounds can not fit as well as compound **1**. The conforma-

tional analysis was thus performed on the six cardiac glycosides.

On the structure optimizing calculations, the resulting minimum energy geometry generally depends on the starting geometry.[9] Hence, all possible rotations in the starting geometry must be examined in order to reach the global minimum. Moreover, when the structure of the enzyme's active site is unknown, it is often assumed that even though the bioactive conformation is not the global minimum, they are not very different from each other. Because a compound should be more active if a smaller energy is required to attain the correct spatial relationship to the enzyme.[10]

Since compounds **5** and **6** have a β-axial 1–4 connected sugar chain, the position of the terminal glucose is significantly different from that of the other cardiac glycosides as can be seen in Fig. 2. Therefore, these glycosides can not be recognized by CGH II.

The other cardiac glycosides have an α-equatorial 1–4 connection between the sugars, therefore, they are similar in overall shape. Besides, cellobioside (**2**) and glucodigimethyloside (**3**) have a 3β-equatorial OH group on the inner sugar, and this OH group is expected to form a hydrogen bond with the pyranose oxygen of the terminal glucose. Actually, all conformations of **2** and **3** listed in Table 2 have a short H$\cdots$O distance possible to form a hydrogen bond (seen also Fig. 2). On the other hand, the corresponding OH group of **1** is 3α-axial, therefore, it is too distant from the glucopyranose oxygen to form a hydrogen bond. No conformation of **1** in Table 2 has the possibility of a hydrogen bond. The terminal glucose of **1** can then easily take an essential position to form the effective complex with CGH II. Contrarily, the intramolecular hydrogen bond of **2** and **3** should restrict the movement of the terminal glucose and, therefore, should prevent the formation of the enzyme-substrate complex. Accordingly, the reaction rate must decrease.

Glucodigimethyloside (**4**) has the same sugar chain as **1**, so they can take the same conformation. Compound **4**, however, has an OH group in position 16 of the gitoxigenin framework. This OH group may cause a crucial steric hindrance to the enzyme active site. Consequently, the reaction rate of **4** must slow down.

In conclusion, the orientation of the terminal glucose with respect to the genin group must play an important role in the formation of the enzyme-substrate complex. A detailed knowledge of the properties and substrate preferences of the enzymes involved in the glycoside formation and hydrolysis will help us to understand the cardenolide pathway, its regulation and the biosynthetic relationship among the individual cardenolides.[11] Our present work contributes to the understanding of the structure and properties of the cardenolide glucohydrolase.

References and Notes

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