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Inversion of the anomeric configuration of the transferred sugar during inactivation of the macrolide antibiotic oleandomycin catalyzed by a macrolide glycosyltransferase

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Abstract Macrolides are a group of antibiotics structurally characterized by a macrocyclic lactone to which one or several deoxy-sugar moieties are attached. The sugar moieties are transferred to the different aglycones by glycosyltransferases (GTF). The OleI GTF of an oleandomycin producer, *Streptomyces antibioticus*, catalyzes the inactivation of this macrolide by glycosylation. The product of this reaction was isolated and its structure elucidated. The donor substrate of the reaction was UDP-α-D-glucose, but the reaction product showed a β-glycosidic linkage. The inversion of the anomeric configuration of the transferred sugar and other data about the kinetics of the reaction and primary structure analysis of several GTFs are compatible with a reaction mechanism involving a single nucleophilic substitution at the sugar anomeric carbon in the catalytic center of the enzyme.

Key words: Glycosyltransferase; Macrolide; Oleandomycin

1. Introduction

Macrolides constitute a group of antibiotics mainly active against Gram-positive bacteria. They have important clinical applications in the treatment of bacterial infections. Their structures are integrated by a macrocyclic lactone to which one or several sugar moieties are attached. These carbohydrate ligands are, in many cases, unusual, and they serve as molecular recognition elements critical for biological activity. The suppression of these ligands produces abolition or a dramatic decrease in activity. In the same way, variations in the sugar composition of these molecules, as changes or additions, can also produce important effects, varying from increase of activity to its total suppression. Because of these considerations, many studies have been performed about the biosynthesis of these molecules in order to apply the acquired knowledge in the emerging field of combinatorial biosynthesis, using them as source to obtain new unnatural compounds susceptible to possess interesting applications. The sugar moieties are transferred to the different aglycones as late steps during the biosynthesis by means of glycosyltransferases (GTF). Important efforts have been carried out on the study of these enzymes in order to apply them for combinatorial biosynthesis. During the last years, several genes encoding GTFs involved in the transfer of sugars to various aglycones have been cloned and sequenced, and their genetic relations analyzed [1–11]. However, because of the unavailability of substrates for these reactions, biochemical information about these enzymes is scarce.

Most macrolides are produced by streptomycetes. The biosynthesis of a potential lethal antibiotic by these microorganisms requires the existence of a self-resistance mechanism in the producing microorganism to avoid cell suicide. One of the resistance mechanisms described in macrolide producing organisms is the existence of intracellular GTF [1,2,9]. These enzymes inactivate macrolides by glycosylation of a hydroxyl group present in one of the 6-deoxy-hexoses attached to the macro-lactone ring [12,13]. This enzymatic activity was initially found in the non-macrolide producer strain *Streptomyces lividans* [1] and in the oleandomycin (OM) producer *Streptomyces antibioticus* [12]. Afterwards, it has been found that such enzyme activity is also present in 15 out of 32 streptomycetes producers of different polyketide antibiotics [14]. The OM producer contains a second enzyme activity that converts the inactive glycosylated OM (Glc-OM) into active antibiotic by removing the glucose moiety from the inactive compound [12]. This glycosidase has been purified from the culture supernatant of this microorganism [15]. Consequently, *S. antibioticus*, which possesses ribosomes that are sensitive to OM all along the cell cycle and even during the production phase of the antibiotic [16], might use this inactivation/reactivation system as a self-resistance mechanism to survive during the biosynthesis of the drug. In the OM biosynthetic gene cluster of *S. antibioticus* the gene (*oleI*) that encodes the OM GTF (OGT) has been identified [9].

Analysis of different GTFs and multiple sequence alignments have shown a great similarity between all known GTFs, including OGT. The differences observed in the primary structure of these enzymes become even diminished when predictive protein structure analysis as multiple predicted secondary structure alignments or hydrophobic cluster analysis are performed (L.M. Quirós, unpublished). This is a good support for a common evolutionary origin of all these proteins, and suggests that all of them could share analogous three-dimensional fold and chemical and kinetic mechanisms.

OGT is an enzyme that catalyzes the transfer of a glucose moiety from UDP-glucose (UDP-Glc) to OM. Both substrates of the reaction are available, what makes this enzyme a good candidate to get further into the biochemical knowledge of this group of enzymes. OGT has been purified and the kinetics

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of the reaction analyzed [17]. Interestingly, in the OM producer organism, another GTF gene (*oleD*) encoding a GTF able to transfer a glucose moiety from UDP-Glc to many macrolides and located outside the OM gene cluster has been cloned [2]. This enzyme has been recently purified and the kinetics of the reaction shown to be analogous to that of OGT [18], what agrees with the hypothesis mentioned above.

In this paper we report the structure determination of the product of the reaction catalyzed by OGT, showing an inversion of the anomeric configuration of the transferred glucose, and correlating this observation with a probable reaction mechanism for this group of enzymes.

2. Materials and methods

2.1. Materials

OM and UDP-Glc were purchased from Sigma. UDP-[6-^{3}H]glucose (specific activity 17.5 Ci mmol^{-1}) was from Amersham. Acetonitrile (HPLC grade) was from Merck. All other chemicals were obtained from commercial sources and were of analytical grade.

2.2. Purification of the OGT

The purification of the OGT encoded by the *oleI* gene was performed as previously described [17].

2.3. Purification of the product of the reaction

OM glycosylation was performed during 12 h at 30°C in a reaction mixture containing 6 mM OM, 9 mM UDP-Glc and 25 μg of pure OGT in Tris–HCl buffer 25 mM pH 8.0. The total volume of the reaction was 5 ml. The reaction was monitorized adding to the mixture a small amount of UDP-[^{3}H]Glc (5 μCi), extracting small aliquots (25 μl) at different times and determining the incorporation of radioactivity into OM as previously described [17]. The OM-Glc was extracted and purified as previously described [18].

2.4. NMR spectroscopy

NMR spectra were recorded at 298 K on a Bruker AMX 400 spectrometer operating at 400.13 MHz and 100.61 MHz for ^{1}H and ^{13}C, respectively, using a 5 mm OXI ^{1}H/^{13}C/^{15}N/^{31}P reverse probe including a z-gradient coil. CD_3OD was used as solvent and chemical shifts were referenced internally to tetramethylsilane (TMS). Sample concentration was 0.1 mM. Pulse widths were 8.5 μs/13.7 μs for ^{1}H/^{13}C at an attenuation level of 3 dB on both channels. A 5% sinus truncated shaped pulse gradient of 1 ms was used with values 5:3:4 for both gs-HMQC and gs-HMBC. Standard Bruker software has been used to acquire and process the ^{13}C DEPT, gs-HMQC, and gs-HMBC spectra. Selected parameters for ^{1}H, ^{13}C 2D correlations: sweep width 2400 Hz for ^{1}H and 19 000 Hz for ^{13}C; 2048×512 data set; pre-acquisition delay 2 s; number of scans 16–32; magnitude mode; data processing using zero filling in the F1 domain and shifted sinus-bell apodization of factor 0 in both dimensions. For HMQC GARP decoupling of ^{13}C was used during acquisition.

3. Results and discussion

The structure of the product of the reaction catalyzed by OGT is unknown, although some indirect approaches have been described [12]. To elucidate the structure of this compound, we purified the enzyme, performed glycosylation reactions and purified several miligrams of the product of the reaction as described in Section 2. The first evidence for the glycosylation in the NMR spectra was the appearance of six additional ^{13}C NMR signals respective to the initial OM substrate. The rest of the signals presented no significant changes with respect to OM, which suggests the same macrocyclic structure plus a new sugar ring. The structural characterization of the compound was performed by the complete assignment of all the ^{1}H and ^{13}C NMR spectral signals. For this

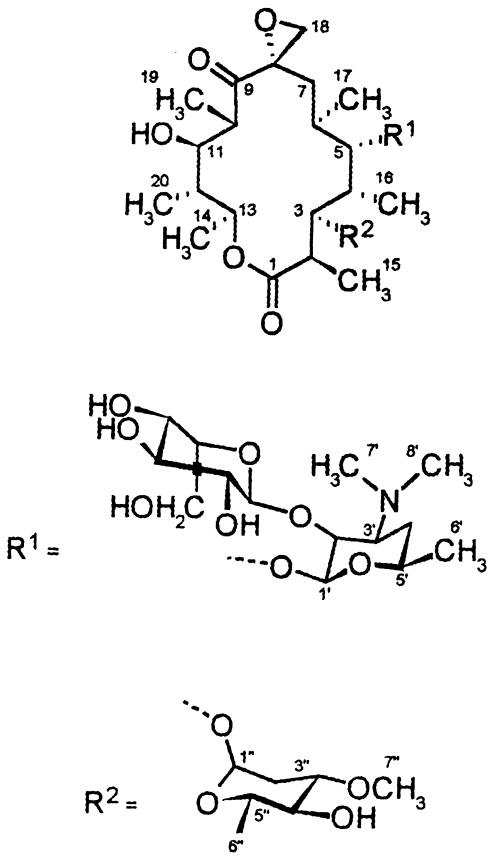

Fig. 1. Chemical structure of Glc-OM.

purpose, apart from the ^{1}H and ^{13}C NMR spectra, other experiments as DEPT, ^{1}H, ^{13}C HMQC and HMBC were necessary. The strategy of the work was to establish in the HMBC spectrum the connectivity of a known carbon with protons separated by two or three bonds and then to find out in the HMQC spectrum the direct response via $^1J_{CH}$ of these protons with their corresponding carbon nuclei. The most evident starting point for the characterization are the ^{13}C carbonyl signals from the C1 (δ 178.82) and C9 (δ 210.56) (Table 1) macrocyclic ring carbons, as they showed the highest unshielding in the ^{13}C NMR spectra. The HMBC spectrum showed correlation peaks through 2 or 3 bonds between C1 and the proton signals H2, H3, H13 and $H_3$15, while C9 correlates with H7, H10, H11, H18 and $H_3$19. The rest of the macrocyclic ^{1}H and ^{13}C signals could be sequentially assigned using the information contained in the HMBC spectrum. The identification of the sugar moieties came from the correlation peaks seen in the same HMBC experiment between protons H5 and H3 with the anomeric carbons C1′ and C1″, respectively. From these starting points the rest of the sugar rings could be easily assigned using the same process discussed above. The direct evidence for the glycosylation position came from the cross peak found between H2′ and C1‴, and between H1‴ and C2′. The conformation of the sugar rings has been deduced from the scalar coupling constants of the protons, as is shown in Fig. 1. According to the fact that changes in the sugar composition of these molecules can produce important changes in biological activity, this Glc-OM molecule is unable to act on the ribosomes, and therefore it can not inhibit protein biosynthesis [9].

Table 1
^{1}H and ^{13}C NMR data of Glc-OM in CD_3OD at 25°C

Site	δ ^{13}C (ppm)	δ ^{1}H (ppm)	Multiplicity	*J*(H, H) (Hz)
1	178.82	–		
2	46.20	2.73	m	
3	80.39	3.81	dd	1.5, 4.6
4	46.47	1.83	m	
5	83.59	3.49	d	9.8
6	34.91	1.89	m	
7	33.59	1.58	dd	10.8, 15.3
		2.50	dd	4.2, 15.3
8	65.32	–		
9	210.56	–		
10	46.50	3.17	dc	1.7, 6.8
11	71.29	3.76	dd	1.7, 9.4
12	43.27	1.66	ddc	1.8, 6.9, 9.4
13	72.21	5.32	dc	1.8, 6.6
14	18.59	1.28	d	6.6
15	13.89	1.16	d	7.2
16	10.46	1.18	d	7.1
17	20.00	1.13	d	6.7
18	50.90	2.71	d	5.0
		2.76	d	5.0
19	8.75	0.98	d	6.8
20	10.11	0.98	d	6.9
1′	104.00	4.34	d	7.3
2′	82.61	3.35	dd	7.3, 10.4
3′	66.91	2.78	m	
4′	30.57	1.27	m	
		1.78	m	
5′	70.10	3.55	m	
6′	21.82	1.21	d	6.1
7′, 8′	41.06	2.27	s	
1″	100.05	4.95	dd	1.2, 3.7
2″	35.84	1.44	ddd	3.7, 11.7, 12.7
		2.37	ddd	1.2, 4.6, 12.7
3″	79.39	3.41	ddd	4.6, 8.9, 11.7
4″	77.68	3.02	dd	8.9, 9.0
5″	70.89	3.69	m	
6″	18.99	1.24	d	6.2
7″	57.66	3.42	s	
1‴	107.77	4.40	d	7.7
2‴	76.81	3.22	dd	7.7, 9.0
3‴	78.61	3.35	m	
4‴	72.10	3.28	m	
5‴	78.43	3.29	m	
6‴	63.66	3.67	dd	5.5, 11.7
		3.84	dd	2.3, 11.7

Interestingly, the coupling constant between H1‴ and H2‴ is 7.7 Hz, only compatible with an axial position of both protons on the sugar ring. This result implies that the glycosidic linkage of the glucose is β. Considering that the substrate of the reaction was UDP-α-D-glucose, an inversion of the anomeric configuration occurred during the reaction. It has been pointed out that the occurrence or non-occurrence of an inversion in an enzyme reaction may afford evidence of the mechanism of the reaction [19]. If the reaction involves only one transfer of the group, the result will be an inversion; if it involves two successive transfers, the effects will cancel and no inversion will be observed. This inversion can only affect the particular atom at which the transfer occurs. The two possibilities have been described for other types of GTFs not involved in macrolide biosynthesis [20–22]. Both mechanisms are summarized in Fig. 2. In the inverting mechanism, a single nucleophilic substitution at the sugar anomeric carbon leads to the formation of a β-linkage from an α-linked donor. The retaining mechanism involves the transient formation of a glycosyl enzyme and its subsequent addition to the acceptor,

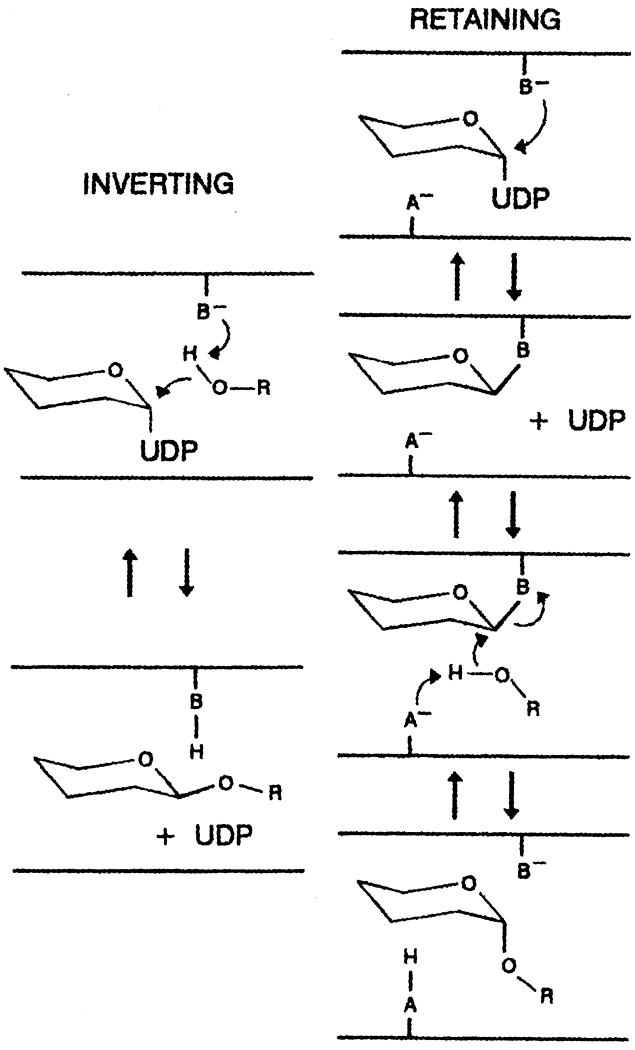

Fig. 2. Proposed mechanisms for retaining and inverting GTF.

resulting in the formation of an α-linkage from an α-linked donor. The retaining mechanism can proceed through oxocarbenium ion-like transition states [22], or may follow a concerted reaction mechanism, through a transition state which avoids formation of the oxocarbenium ion reaction intermediate [23]. Additional considerations can be made using the kinetic information previously reported for OGT [17]. The retaining mechanism is more compatible with a reaction in which the enzyme reacts with one substrate to give a covalently modified enzyme and releases one product, reacting then with the second substrate. The reaction would proceed in a compulsory order through a series of binary complexes, and no ternary complex would be formed. Nevertheless, the kinetic mechanism described for OGT implies the binding of the substrates to the enzyme in a compulsory order, first OM and then the UDP-Glc to form a ternary complex in which the exchange reaction takes place; then, the release of the products also occurs in a compulsory order, UDP first fol-

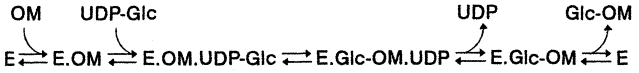

Fig. 3. Kinetic mechanism of OGT.

lowed subsequently by Glc-OM (Fig. 3). The same mechanism has been described for the other GTF isolated from *S. antibioticus* using the macrolide lankamycin as substrate [18]. These results are more in agreement with a mechanism that would involve the inverting reaction, implying a single nucleophilic substitution in a ternary complex at the catalytic center of the enzyme.

Another interesting aspect of the mechanism is that it implies the presence of a nucleophile at the catalytic center. Recently, kinetic studies at different pHs for the GTF encoded by *oleD* from *S. antibioticus* have determined molecular pK values for V and V/K_m placed between 6.6 and 6.8 [18], values within the pK range described for histidine when present at the active site [24], and suggesting that this amino acid could be involved in the catalytic process. Mechanisms for GTFs using oligosaccharides as donor have been determined by labeling, crystallography and kinetic analysis of several enzymes, showing their active sites a pair of carboxylic acids that play key roles in the mechanisms [22]. Nevertheless, much less information is available on nucleotide phospho-sugar dependent GTFs, as those implied in the biosynthesis of macrolides and other polyketide molecules. The imidazole side chain of histidine is a ternary amine in which the atoms bound to the nitrogen are held back in the five-membered ring, thus avoiding steric hindrance. This amine has a pK_a value near 7, what makes it extremely effective as a nucleophilic catalyst [25]. In addition, analysis of the amino acid sequences of several GTFs involved in the biosynthesis of polyketides showed a very well conserved region including one or two histidine residues depending on the GTF (Fig. 4). These considerations again correlate very well with the proposed mechanism, and suggest that the amino acid histidine present at this conserved region could play an important role in the catalytic activity of the enzyme. However, not so well conserved regions including carboxylic acids can be detected in the alignments for the different macrolide GTFs, although histidine residues have also been shown to be important in terms of substrate binding and transition state stabilization in some oligosaccharide-dependent GTFs.

In conclusion, all the results and considerations given above support a chemical mechanism for the OGT in which an inversion at the glucose anomeric carbon takes place, leading to the formation of a β-linkage from an α-linked donor. This mechanism is supported by the elucidated structure of the reaction product, but it is also consistent with the kinetic mechanism described for the macrolide GTFs codified by the *oleI* and *oleD* genes from *S. antibioticus*. Furthermore, it could also be supported by the kinetic studies at different pHs performed for the GTF encoded by *oleD*, and with the existence of a well-conserved region including one or two histidines in all GTFs so far described involved in the biosynthesis of polyketides. The similarities in the sequences and predicted structures from these enzymes makes agreeable to extend this mechanism to this family of enzymes.

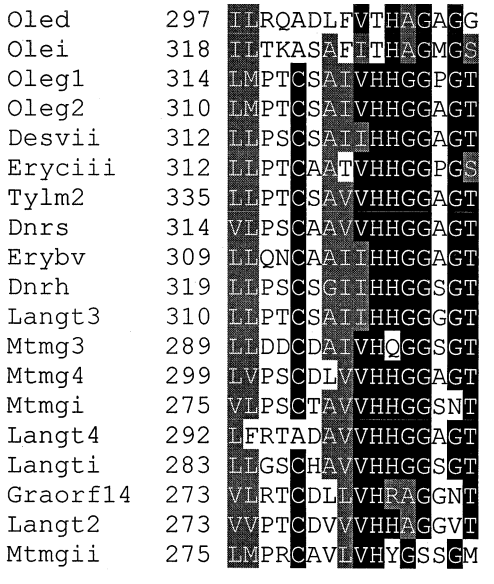

Fig. 4. Alignment of a conserved region in several GTF involved in the biosynthesis of different polyketide antibiotics

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