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UDP-L-arabinose-hydroxyproline-*O*-glycosyltransferases in *Volvox carteri*

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Hydroxyproline-containing peptides of different length and amino acid sequence have been used to demonstrate UDP-L-arabinose-hydroxyproline-*O*-glycosyltransferases in a crude microsomal fraction from the green alga *Volvox carteri*. The formation of *O*-glycosidic linkages by transfer of UDP-activated arabinose to the side chain of hydroxyproline was concluded from the resistance of the glycopeptides under the basic conditions of β-elimination and their susceptibility to hydrolysis by trifluoroacetic acid. This treatment yielded arabinose as the only cleavage product. Arabinose transfer to the various peptide substrates was found to be stimulated by low concentrations of detergent, to require divalent cations and to proceed optimally at pH values around 7.0. The smallest arabinose acceptor peptide was the tripeptide Tyr-Hyp-Lys. The glycosyl acceptor effectivity increased with increasing numbers of repeated hydroxyproline residues, suggesting that hydroxyproline clusters critically affect substrate recognition by the *Volvox* transferase(s).

Hydroxyproline cluster; *O*-Arabinosylation; UDP-L-arabinose-hydroxyproline-*O*-glycosyltransferase; (*Volvox carteri*)

1. INTRODUCTION

In vitro studies with model peptides of defined composition and amino acid sequence have provided significant insights into structural features required for *N*- and *O*-glycosylation of proteins [1,2]. By this approach, it has been proved that triplet sequences of the type Asn-Xaa-Thr or Asn-Xaa-Ser represent a necessary, though in itself not sufficient prerequisite for *N*-glycosylation as previously predicted by Marshall [3] on the basis of known glycoprotein sequence data. Comparable sequence homologies for *O*-glycosyl transfer to serine or threonine side chains have not yet been detected. Although proline residues in the vicinity of these acceptor amino acids appear to favour *O*-glycosylation, it is postulated that accessibility of the potential acceptor site rather than a specific signal sequence is the key for *O*-glycosylation [4–6].

The green alga *Volvox carteri* synthesizes *O*-arabinosides in cell wall components and specific glycoproteins. It seems to be a useful organism for studying these processes as the alga was recently found to contain hydroxyproline- and arabinose-rich glycoproteins [7]. Using hydroxyproline-containing peptides as probes, we have investigated whether *V. carteri* possesses *O*-glycosyltransferase activities for the formation of such linkages between arabinose and hydroxyproline. We present evidence that *O*-glycosyltransferase activities are indeed present in *Volvox* microsomes. In addition, we show that the glycosyl acceptor properties of the hydroxyproline peptide substrates increase with their length and the number of re-

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Abbreviations: TFA, trifluoroacetic acid; Hyp, hydroxyproline; Mops, 3-(*N*-morpholino)propanesulfonic acid; Boc-, *t*-butoxycarbonyl-; Xaa, 'variable' amino acid

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peated imino acid residues. The data suggest that clustered hydroxyprolines represent the structural motif for recognition by the arabinosyltransferase.

2. MATERIALS AND METHODS

2.1. *Materials*

The materials used and their sources were as follows: UDP-[^{14}C]arabinose (spec. act. 183 Ci/mol), NEN; UDP-[^{14}]GlcNAc (spec. act. 346 Ci/mol), GDP-[^{14}C]mannose (spec. act. 155 Ci/mol), The Radiochemical Centre, Amersham; Triton X-100, Lubrol PX, Sigma, Taufkirchen; trifluoroacetic acid, α-*N*-Boc-amino acids, L-hydroxyproline, α-*N*-dansylarginine, Fluka; Bio-Gel P2 and Bio-Beads S-X1 (chloromethylated), Bio-Rad Laboratories; cellulose plates, Schleicher & Schüll. All other chemicals were from commercial sources and of the highest purity available. α-L-Arabinofuranosidase from *Monilinia fructigena* was kindly provided by Dr M. Sinnott, Bristol.

2.2. *Peptide synthesis*

Peptides were synthesized by the solid-phase method as described by Merrifield [8] and Erickson and Merrifield [9]. L-Hydroxyproline was attached as the α-*N*-Boc-*O*-benzyl derivative which was prepared as in [10]. Peptides were cleaved from the resin with HBr in TFA and purified by gel chromatography on Bio-Gel P4 with 1 M acetic acid as the solvent. Their purity was checked by amino acid analysis and TLC on silica gel with *n*-butanol/acetic acid/water (4:1:1, by vol.) and *n*-propanol/acetic acid/water (3:3:2, by vol.) as the solvents.

2.3. *Standard O-glycosylation assay*

A typical incubation mixture for measuring peptide glycosylation contained the following components in a total volume of 100 μl: 200000 cpm UDP-[^{14}C]arabinose in 23 μl of 50 mM Mops buffer, pH 7.0, 5 μl of 100 mM $MnCl_2$ in water, 2 μl of 5% Lubrol PX in 50 mM Mops buffer, pH 7.0, 10 μl of aqueous stock solutions of peptides in water, pH 7.0, and 60 μl of *Volvox* microsomes. At the indicated intervals, the reactions were terminated by the addition of 0.5 ml methanol. The precipitates were centrifuged off and the supernatants containing the ^{14}C-glycopeptides analysed by electrophoresis at pH 1.9 in 1 M formic acid on cellulose plates. α-*N*-Dansylarginine was included as cationic marker.

2.4. *Methods for the analysis of ^{14}C-glycopeptides*

2.4.1. β-Elimination

This was carried out by incubating the purified ^{14}C-glycopeptides in 0.1 M NaOH for 48 h at 40°C. The cleavage of *O*-glycosidic linkages was monitored by electrophoresis at pH 1.9 and/or Bio-Gel P2 chromatography.

2.4.2. Trifluoroacetic acid hydrolysis

The purified ^{14}C-glycopeptides were treated with 2 N TFA in water for 3 h at 120°C. After drying cleavage products were analysed by electrophoresis at pH 1.9 and paper chromatography with pyridine/methyl acetate/water (3.6:1.0:1.08, by vol.; upper phase) as the solvent.

2.5. *General procedures*

Volvox cultures (female strain HK 10) were grown axenically in Provasoli *Volvox* medium as described in [11]. A crude microsomal fraction, used as the enzyme source, was prepared as in [12]. Radioactive zones on the chromatograms were detected by radioscanning on a Berthold LB 2722 radioscanner. ^{14}C-glycopeptides were quantified after elution with water/methanol (1:1, by vol.) by counting in a Delta 300 liquid scintillation counter (Searle Analytic) using Bray's scintillation cocktail [13]. Amino acid analyses were carried out on a Beckman amino acid analyser after hydrolysis of peptides with 5.7 N HCl for 20 h at 110°C.

3. RESULTS AND DISCUSSION

3.1. *Transfer of arabinose onto hydroxyproline-containing peptides and characterization of radiolabelled reaction products*

Table 1 summarizes the amino acid sequence of the model peptides used in this study. With the exception of VIII, the peptides contain one or more hydroxyproline residues as potential sugar acceptor sites. In addition, most of them contain the basic amino acid lysine, the positively charged side chain of which was expected to facilitate separation of the glycopeptides under the acidic conditions of electrophoresis.

Incubation under standard assay conditions of

Table 1

Amino acid sequence and acceptor properties of peptides

Peptide	Amino acid sequence	*O*-Glycosyl acceptor	K_m (mM)
I	Tyr-Hyp-Lys	+	3.5
II	Ser-Hyp-Lys	+	n.d.
III	Tyr-Ser-Hyp-Lys	+	2.8
IV	Tyr-Ala-Hyp-Lys	+	n.d.
V	Tyr-Ser-Hyp_2-Lys	+ +	1.8
VI	Tyr-Ser-Hyp_4-Lys	+ + +	0.8
VII	Gly-Pro-Hyp	no acceptor	
VIII	Tyr-Ser-Pro-Lys	no acceptor	

Glycosyl acceptor properties were measured as described in section 2. n.d., not determined; (+, + +, + + +) relative acceptor capability

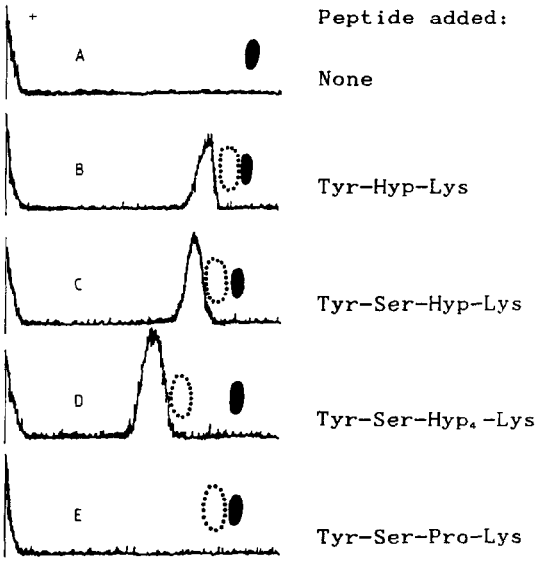

Fig.1. Analysis of ^{14}C-labelled reaction products by electrophoresis. A particulate membrane fraction from *V. carteri* was incubated under standard assay conditions in the presence of 200 000 cpm UDP-[^{14}C]arabinose and 6 mM peptides as indicated. After 2 h at room temperature, the ^{14}C-labelled reaction products were separated by electrophoresis at pH 1.9 on cellulose plates and identified by radioscanning. The electrophoretogram shows the cationic region only. α-DNS-arginine was included as 'front marker' (filled areas). The positions of unglycosylated peptides are indicated by the areas enclosed by dotted lines.

Volvox crude microsomes in the presence of UDP-[^{14}C]arabinose and various hydroxyproline-containing peptides (Tyr-Hyp-Lys; Tyr-Ser-Hyp-Lys; Tyr-Ser-Hyp-Hyp-Hyp-Hyp-Lys) resulted in the formation of radiolabelled products which, on electrophoresis at pH 1.9, had a somewhat lower mobility than the corresponding peptides present in the incubation assays (fig.1, lanes B–D). These ^{14}C-labelled reaction products were seen neither in the control incubations (lane A) nor in the presence of the hydroxyproline-free tetrapeptide Tyr-Ser-Pro-Lys (lane E). UDP-[^{14}C]arabinose could not be substituted by other sugar nucleotides such as UDP-[^{14}C]GlcNAc or GDP-[^{14}C]mannose (not shown).

In order to characterize the nature of the radiolabelled glycopeptides, the corresponding ^{14}C bands obtained after electrophoresis were eluted and the eluate applied to a Bio-Gel P2 column. A typical chromatographic pattern from incubations with peptide I is shown in fig.2. The ^{14}C-glycopeptide eluted at a molecular mass somewhat larger than that of the unglycosylated acceptor Tyr-Hyp-Lys itself. The difference in their molecular masses corresponds to one monosaccharide unit, as follows from the elution behaviour of mono- and unglycosylated glycopeptide standards. This points to a transfer of one arabinose residue to the acceptor tripeptide.

The ^{14}C-glycopeptides obtained from incubations with various hydroxyproline-containing acceptor peptides were stable under the basic conditions of β-elimination, a procedure which is known to cleave *O*-glycosidic linkages between sugar and serine/threonine residues. By contrast, treatment of the ^{14}C-glycopeptides with 2 M TFA for 3 h at 120°C led to a radioactive cleavage product which eluted on Bio-Gel P2 chromatography as a monosaccharide. Paper chromatography with monosaccharide standards identified this acidic ^{14}C-labelled cleavage product as arabinose. The various observations clearly demonstrate the presence in *Volvox* microsomes of *O*-glycosyltransferases capable of synthesizing *O*-glycosidic linkages between arabinose as the sugar component and hydroxyproline as the acceptor. Attempts to cleave the arabinosyl-*O*-hydroxyproline linkage in the ^{14}C-glycopeptides with a specific α-L-arabinofuranosidase from *Monilinia fructigena* failed, suggesting the β-configuration

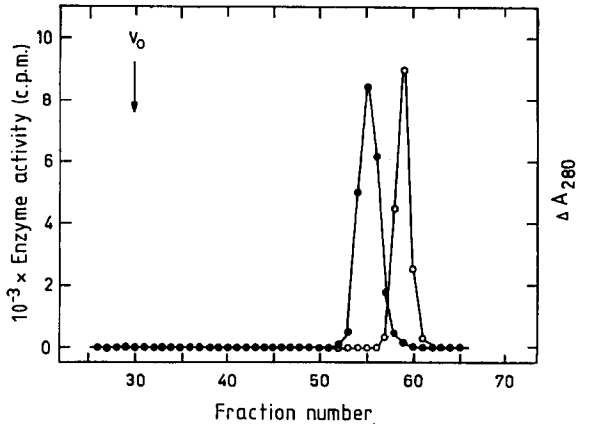

Fig.2. Bio-Gel P2 chromatography of radiolabelled glycopeptide. The ^{14}C-glycopeptide synthesized in the presence of the Tyr-Hyp-Lys tripeptide was eluted with methanol/water (1:1; v/v) from the cellulose plate after electrophoresis and cochromatographed on a Bio-Gel P2 column (1.5 × 90 cm) wity Tyr-Hyp-Lys as standard. The unglycosylated peptide was identified by its UV absorbance at 280 nm, the ^{14}C-glycopeptide by liquid scintillation counting of aliquots of the fractions [(○), unglycosylated tripeptide, (●) ^{14}-C-glycopeptide].

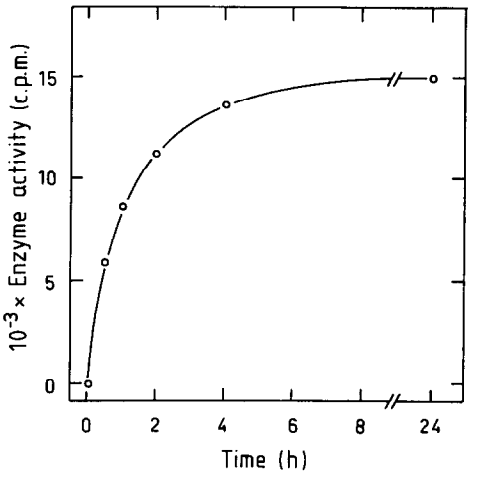

Fig.3. Time course of arabinose transfer from UDP-arabinose to the Tyr-Hyp-Lys tripeptide. *Volvox* microsomes were incubated under standard assay conditions in the presence of 6 mM Tyr-Hyp-Lys. After the indicated times, the reactions were terminated and the ^{14}C-glycopeptide isolated as described in section 2.

for the glycosidic bond. This preliminary interpretation is in accordance with analytical data, e.g. on the structure of lectins from potato tubers and thorn apple seeds by which a β-linkage between arabinose and hydroxyproline has been demonstrated [15].

3.2. *Enzymatic properties and substrate specificity of UDP-arabinose-hydroxyproline-O-glycosyltransferase from* Volvox

The time dependence of arabinose transfer to the Tyr-Hyp-Lys acceptor peptide is shown in fig.3. The deviation from linearity observed after longer incubation times is probably due to substrate depletion caused by the presence of pyrophosphatase and phosphatase activities in the crude microsomal fraction as indicated by the formation of [^{14}C]arabinose and [^{14}C]arabinose-1-phosphate during incubations. The cleavage of the sugar nucleotide donor could be partially inhibited by adding ADP(NH)P.

Although significant amounts of ^{14}C-glycopeptides were synthesized in the absence of detergents, addition of 0.2% Triton X-100 or Lubrol PX to the incubation medium stimulated glycopeptide formation about 2-fold, whereas higher concentrations of detergent were found to be inhibitory and resulted in a gradual and finally complete loss of transferase activity (fig.4). The enhancement of ^{14}C-glycopeptide formation in the presence of low detergent may be caused by greater accessibility of the enzyme when the microsomal vesicles are disrupted, or may indicate that the transferase is membrane-bound.

The pH optimum of the *Volvox* O-glycosyltransferase, measured with the tripeptide Tyr-Hyp-Lys as acceptor, was found to be close to pH 7.0 with half-maximal activity at pH 6.2 and 7.4, respectively (fig.5). The enzyme was partially inhibited by 1 mM EDTA. Activity could be restored, however, by the addition of Mn^{2+} or Mg^{2+}, indicating that the *Volvox* transferase requires cations.

Arabinosylation of the various hydroxyproline-containing peptides was concentration-dependent. The glycosyl acceptor properties improved generally with increasing chain length and increasing number of clustered hydroxyproline residues as follows from the decrease in K_m for peptides I, III, V and VI (see table 1). By contrast, neither the size nor the number of hydroxyproline residues affected V_{max} significantly, indicating that hydrox-

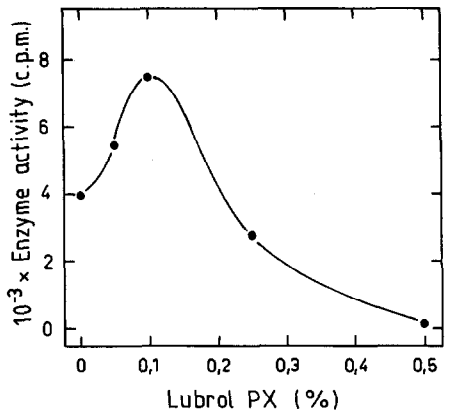

Fig.4. Detergent dependence of UDP-arabinosyl-hydroxyproline-*O*-glycosyl-transferase from *Volvox*. Crude *Volvox* microsomes were incubated under standard assay conditions in the presence of 6 mM Tyr-Hyp-Lys and varying concentrations of Lubrol PX. ^{14}C-glycopeptide formation was measured as described in section 2.

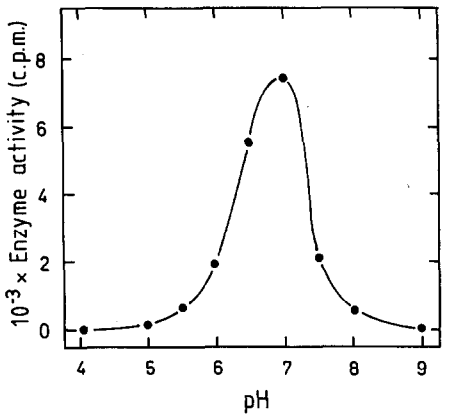

Fig.5. pH dependence of UDP-arabinosyl-hydroxyproline-*O*-glycosyl-transferase. Transferase activity was measured by incubating *Volvox* microsomes in the presence of 6 mM Tyr-Hyp-Lys at the indicated pH values and the ^{14}C-glycopeptide analysed and quantified as described in section 2.

yproline clusters in the vicinity of the acceptor imino acid are important for recognition and binding rather than for modulating the catalytical properties of the arabinosyltransferase. The tripeptide Gly-Pro-Hyp was not glycosylated under the standard assay conditions. This suggests that a negative charge at the unblocked C-terminus of the acceptor imino acid is unfavourable to the transferase. Corresponding observations were made in in vitro model studies on the *N*-glycosylation of Asn-Xaa-Thr/Ser triplets in liver [14] and the *O*-mannosylation of threonine/serine-containing peptides in yeast [4,6].

3.3. *Conclusions*

The in vitro studies described in this paper, demonstrate that particulate fractions of the green alga *V. carteri* contain *O*-glycosyltransferase activities which catalyse the formation of *O*-glycosidic linkages of presumably β-configuration between arabinose and the side chain of hydroxyproline. The synthesis of this type of glycosidic bond is concluded from the following observations:

(i) ^{14}C-glycopeptides are observed only in incubations carried out in the presence of hydroxyproline-containing peptides;

(ii) the glycosidic bond is stable under conditions of β-elimination, but hydrolysed with TFA, the latter treatment yielding [^{14}C]arabinose as the only cleavage product;

(iii) UDP-arabinose as the glycosyl donor cannot be replaced by other sugar nucleotides;

(iv) the ^{14}C-glycopeptides are not susceptible to hydrolysis by α-L-arabinofuranosidase.

The *Volvox O*-glycosyltransferase catalysing the synthesis of the arabinose-hydroxyproline linkage appears to be a membrane-bound enzyme and to require divalent cations for activity. The minimum substrate requirement for arabinosyl transfer is the presence of a hydroxyproline residue in a tripeptide. Peptides having the acceptor imino acid located at the C- (and probably N-) terminus are not glycosylated. The acceptor properties, expressed by the K_m values of the peptides, improve significantly when the number of hydroxyproline residues is increased supporting the view that hydroxyproline clusters, most likely due to their specific structure-forming potential, contribute critically to the formation of the sugar-attachment site and its recognition by the arabinosyltransferase. This interpretation is in agreement with results of recent studies on the amino acid sequence of a hydroxyproline/arabinose-rich *Volvox* glycoprotein occurring in the morphogenic process of inversion [7]. Here, extended homologous sequence regions containing more than six hydroxyproline residues have been identified. We are currently

synthesizing peptide substrates having more extended regions of clustered and variable hydroxyproline sequences in order to analyse this structural aspect in more detail.

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