# Crystal Structure of Uronate Dehydrogenase from Agrobacterium tumefaciens\*<sup>S</sup>

Received for publication, April 26, 2011, and in revised form, June 2, 2011 Published, JBC Papers in Press, June 15, 2011, DOI 10.1074/jbc.M111.254854

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Uronate dehydrogenase from Agrobacterium tumefaciens (AtUdh) belongs to the short-chain dehydrogenase/reductase superfamily and catalyzes the oxidation of D-galacturonic acid and D-glucuronic acid with NAD<sup>+</sup> as a cofactor. We have determined the crystal structures of an apo-form of AtUdh, a ternary form in complex with NADH and product (substrate-soaked structure), and an inactive Y136A mutant in complex with NAD<sup>+</sup>. The crystal structures suggest AtUdh to be a homohexamer, which has also been observed to be the major form in solution. The monomer contains a Rossmann fold, essential for nucleotide binding and a common feature of the short-chain dehydrogenase/reductase family enzymes. The ternary complex structure reveals a product, D-galactaro-1,5-lactone, which is bound above the nicotinamide ring. This product rearranges in solution to D-galactaro-1,4-lactone as verified by mass spectrometry analysis, which agrees with our previous NMR study. The crystal structure of the mutant with the catalytic residue Tyr-136 substituted with alanine shows changes in the position of Ile-74 and Ser-75. This probably altered the binding of the nicotinamide end of NAD<sup>+</sup>, which was not visible in the electron density map. The structures presented provide novel insights into cofactor and substrate binding and the reaction mechanism of AtUdh. This information can be applied to the design of efficient microbial conversion of D-galacturonic acid-based waste materials.

D-Galacturonic acid is the main component of pectin, a natural polymer that exists in primary cell walls of terrestrial plants. Citrus peel and sugar beet pulp are cheap raw materials, and both contain a large amount of pectin, which is currently exploited mainly as cattle feed. Pectin has the potential to be an important raw material for biotechnical conversions to fuels and chemicals. The microbial pathways of D-galacturonic acid catabolism have recently been described (1). Two different catabolic pathways, the isomerase pathway and the oxidative pathway, have been found in bacteria. The isomerase pathway (in *Escherichia coli*) converts D-galacturonic acid into pyruvate and D-glyceraldehyde 3-phosphate. The oxidative pathway has been described for *Agrobacterium tumefaciens* and *Pseudomonas syringae* (2, 3). In this pathway, D-galacturonic acid is first oxidized into *meso*-galactaric acid and then converted in the following step to  $\alpha$ -ketoglutarate.

Uronate dehydrogenase (EC 1.1.1.203) is the key enzyme in the oxidative pathway of D-galacturonic acid catabolism in bacteria. The enzyme catalyzes the oxidation of D-galacturonic acid into D-galactaric acid. Uronate dehydrogenases from *A. tumefaciens* (4) and *P. syringae* (5) have been purified and characterized, and the corresponding genes have also been identified (6, 7). *A. tumefaciens* (*Rhizobium radiobacter*) uronate dehydrogenase (AtUdh)<sup>2</sup> is specific for NAD<sup>+</sup> as a cofactor but accepts both D-galacturonic acid and D-glucuronic acid as substrates with similar affinities. AtUdh belongs to the short-chain dehydrogenase/reductase (SDR) superfamily. SDR proteins are NAD(P)(H)-dependent enzymes with a wide spectrum of substrate specificities and also different enzyme classes (21). The sequence identities between the members of the SDR family are low, but they share a similar three-dimensional  $\alpha/\beta$ -structure.

To date, no structural information on uronate dehydrogenase is available. Here, we present the first three-dimensional structure of uronate dehydrogenase, namely AtUdh. We determined the crystal structures of AtUdh in the apo-form and the ternary complex with NADH and product at 1.9 and 2.1 Å resolutions, respectively. In addition, we performed a site-directed mutagenesis study of the catalytic residue Tyr-136 and determined the NAD<sup>+</sup>-bound crystal structure of the inactive mutant Y136A. This crystallographic information has enabled us to identify the active site of the enzyme and the molecular basis for cofactor and substrate recognition. We also propose a structure-based mechanism for the oxidation of D-galacturonic acid. This information can be used to improve the properties of the enzyme, especially the substrate specificity and enzymatic activity.

### **EXPERIMENTAL PROCEDURES**

*Cloning, Expression, and Purification of AtUdh*—Cloning, expression, and purification were performed as described previously (6). Briefly, the N-terminal His<sub>6</sub>-tagged gene was expressed in *Saccharomyces cerevisiae* under the control of the



<sup>\*</sup> This work was supported by the Academy of Finland through the Finnish Centre of Excellence in White Biotechnology-Green Chemistry (Decision 118573).

The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. S1.

The atomic coordinates and structure factors (codes 3RFT, 3RFV, and 3RFX) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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<sup>&</sup>lt;sup>2</sup> The abbreviations used are: AtUdh, *A. tumefaciens* uronate dehydrogenase; SDR, short-chain dehydrogenase/reductase; DesIV, dTDP-glucose 4,5-dehydratase.

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constitutive triose-phosphate isomerase promoter, and AtUdh was purified from the yeast cell extract in a single step using nickel-nitrilotriacetic acid chromatography.

Site-directed Mutagenesis—The  $His_6$ -tagged uronate dehydrogenase gene in the p2159 vector (6) was used as the template for all mutagenesis reactions. A site-directed mutation of tyrosine at position 136 to alanine was introduced using the QuikChange site-directed mutagenesis kit from Stratagene (La Jolla, CA). The mutant was transformed into the *E. coli* XL1-Blue strain, isolated, and sequenced using an ABI PRISM 3100 genetic analyzer (Applied Biosystems, Foster City, CA). Expression and purification of the His<sub>6</sub>-tagged Y136A mutant were carried out in *S. cerevisiae* using essentially the same protocol as for the wild-type enzyme. The activity of the purified mutant enzyme was assayed on D-galacturonic acid at pH 7.5 and 2 °C by following the formation of NADH at 340 nm as described (6).

Inhibition of AtUdh Activity—The effect of sulfate on AtUdh activity was determined by following the change in absorbance at 340 nm in the presence of 1  $\mu$ M to 100 mM ammonium sulfate at 22 °C. In the assay, 1  $\mu$ g of AtUdh was used with 4 mM D-galacturonic acid and 0.5 mM NAD<sup>+</sup> in 50 mM Tris-HCl (pH 7.5) containing 10 mM MgCl<sub>2</sub>.

Size-exclusion Chromatography—Size-exclusion chromatography to analyze the oligomeric state of the AtUdh was performed at 22 °C on a Superdex 200 HR 10/30 column equilibrated in 50 mM sodium phosphate and 150 mM NaCl (pH 8.0) and run at a flow rate of 0.5 ml/min. The column was calibrated with gel filtration standard proteins (GE Healthcare) giving the following elution volumes: ferritin (440,000 Da), 14.3 ml; aldolase (158,000 Da), 15.3 ml; ovalbumin (44,000 Da), 16.5 ml; and ribonuclease A (13,700 Da), 18.0 ml. The void volume of the column was determined by thyroglobulin, which eluted at 8.5 ml. A 250- $\mu$ l sample containing 29  $\mu$ g of AtUdh was loaded onto the column, and the eluate was monitored at 280 nm. Fractions of 0.5 ml were collected, and aliquots from the fractions were analyzed by SDS-PAGE.

Mass Spectrometry-Electrospray ionization MS was performed on a quadrupole ion trap instrument (Esquire 3000 Plus, Bruker Daltonics, Bremen, Germany) operated in a negative-ion mode. This instrument has been described in detail previously (8). D-Galacturonic acid was dissolved in water to prepare a 100 mM stock solution. For electrospray ionization MS, the stock solution was further diluted to 10  $\mu$ M with acetonitrile and directly electrosprayed at a flow rate of 2  $\mu$ l/min. For hydrogen/deuterium exchange, the stock solution was first diluted to 100  $\mu$ M with D<sub>2</sub>O, allowed to stand for 15 min, and further diluted to 10  $\mu$ M with acetonitrile- $d_3$ . For collision-induced dissociation MS/MS spectra, the ions of interest were mass-selected and fragmented using the most appropriate fragmentation amplitudes. For reaction end product analysis, a mixture of 100  $\mu$ M D-galacturonic acid and 180  $\mu$ M NAD<sup>+</sup> was prepared in 1 ml of 10 mM ammonium bicarbonate (pH 8.3), and 2  $\mu$ l of AtUdh (4 mg/ml) was added to the reaction mixture and allowed to stand at room temperature for 30 min. The reaction was stopped by diluting the reaction mixture with acetonitrile (1:10) and directly analyzed. For hydrogen/deuterium exchange, 10 mM ammonium bicarbonate prepared in D<sub>2</sub>O was used instead. The instrument was controlled and the data were processed using Bruker Daltonics Compass 1.1 for Esquire/ HCT software. The spectra were further analyzed with Bruker DataAnalysis 4.0 software.

Crystallization-All crystallization experiments were performed at room temperature using the hanging drop vapor diffusion method. The protein concentration was 4 mg/ml in 50 mM sodium phosphate (pH 8), 150 mM NaCl, and 1 mM β-mercaptoethanol. 1  $\mu$ l of protein solution was mixed with 1  $\mu$ l of reservoir solution and equilibrated against 0.5 ml of reservoir solution. Crystal screening was carried out using Crystal Screen<sup>TM</sup> (Hampton Research). The first crystallization condition consisted of 2.0 M ammonium sulfate and 0.1 M sodium acetate (pH 4.6). To optimize the size of the crystals, the concentration of ammonium sulfate was decreased to 1.8 M. Under this condition, crystals  $0.4 \times 0.4 \times 0.4$  mm in size grew within a few days. These crystals were used for crystal structure determination and for the apo-structure. For the soaking experiments, a second crystallization condition was optimized because only half of the NAD<sup>+</sup> molecule was observed in the cofactor-binding site in the first crystals. The second crystallization condition consisted of 1.8 M ammonium phosphate and Tris-HCl (pH 8.5), and the crystals were isomorphous with the crystals obtained earlier. These latter conditions were also used for crystallization of the Y136A mutant.

The heavy atom derivatives were prepared by soaking crystals in cryoprotectant solution (reservoir solution containing 30% glycerol) containing 10 mM heavy atom for 10 min. For the soaking of crystals, 5 mM NAD<sup>+</sup> and 10 mM D-galacturonic acid was added to the reservoir solution. Crystals were soaked for  $\sim$ 10 min and transferred to cryoprotectant solution containing soaking solution with an additional 30% of glycerol. After a quick soak in cryoprotectant solution, the crystals were cooled in liquid nitrogen.

Data Collection and Processing and Structure Determination-All crystals were frozen in liquid nitrogen after being soaked in cryoprotectant solution, and the data were collected at 100 K. European Synchrotron Radiation Facility (ESRF) beamlines ID14-4 and ID23-1 and DESY beamline X12 were used as x-ray sources. All data sets were indexed, integrated, and scaled using the program package XDS/XSCALE (9). The apo-crystals diffracted to a resolution of 1.9 Å. The crystals belong to the hexagonal space group P6<sub>2</sub>22 (a and b = 164.6 Å and c = 174.8 Å), with a solvent content of 68% and three molecules in the asymmetric unit. The crystal structure of AtUdh was solved by the SIRAS (single isomorphous replacement with anomalous scattering) method using the HKL2MAP graphical user interface (10). Native and derivative data sets were scaled together with SHELXC, and the heavy atom positions were determined by using SHELXD (11). SHELXE was then used to compute the protein phases using a KAu(CN)<sub>2</sub> derivative crystal. The resultant electron density map was used to automatically fit the sequence using Buccaneer (12), which built a total of 796 residues for the three molecules. The remainder of the model was manually built with COOT (13), and the refinement was subsequently performed with PHENIX (14). The structures of AtUdh soaked with NAD<sup>+</sup> and D-galacturonic acid at pH 8.5 and the Y136A mutant soaked with NAD<sup>+</sup> were solved by molecular replacement using the apo-structure as an initial model. Model



#### TABLE 1

#### Data collection and refinement statistics

PDB, Protein Data Bank; r.m.s.d., root mean square deviation.

	Apo-structure	KAu(CN) <sub>2</sub>	NADH and product	Y136A
Beamline	ID14-4	ID14-4	X12	ID23-1
PDB code	3RFT		3RFV	3RFX
Space group	P6,22	P6,22	P6,22	P6 <sub>2</sub> 22
Únit cell (Å)	a  and  b = 164.7, c = 174.8	a  and  b = 164.2, c = 174.5	a  and  b = 165.4, c = 173.6	a  and  b = 165.5, c = 174.1
Resolution (Å)	1.9	1.9	2.1	1.9
High resolution shell (Å)	1.95-1.90	1.95-1.90	2.15-2.10	1.95-1.90
$R_{\rm merge}$ (%)	8.2 (32.9)	6.9 (33.6)	8.0 (36.9)	7.1 (38.2)
Completeness (%)	100 (100)	95.7 (87.0)	99.8 (100)	97.6 (98.9)
Multiplicity	5.7 (5.7)	5.7 (3.8)	11.0 (11.0)	11.0 (11.1)
$I/\sigma I$	18.3 (4.8)	21.5 (4.3)	27.3 (6.8)	23.3 (6.1)
R-factor (%)	15.1		15.7	15.8
$R_{\rm free}$ (%)	17.2		18.9	18.0
No. of atoms	7066		7114	7118
Protein	6175		6132	6111
Water	876		771	896
NADH			132	81
Product lactone			39	
Other	15 (SO <sub>4</sub> )		40 (PO <sub>4</sub> )	30 (PO <sub>4</sub> )
Mean B-factor (Ų)	20.1		23.0	21.4
NADH			24.7	27.4
Product lactone			35.1	
r.m.s.d. bond length (Å)	0.007		0.007	0.007
r.m.s.d. bond angles	1.052°		1.091°	1.052°
Ramachandran plot (%)				
Most favored	91.5		90.8	92.2
Additionally allowed	8.5		9.2	7.8

building was carried out with COOT, and the refinement was performed with PHENIX. The validation of the models was performed with PROCHECK (15). The data collection and refinement statistics are summarized in Table 1.

#### RESULTS

Structure Determination-The protein crystals of AtUdh belong to the space group P6<sub>2</sub>22. There are three AtUdh monomers in the asymmetric unit, and the Matthews coefficient (16) is 3.8 Å<sup>3</sup>/Da, which corresponds to an estimated solvent content of 68%. The apo-structure of AtUdh was solved by x-ray crystallography at 1.9 Å resolution and phased by the SIRAS method with a gold derivative. The final model consists of 813 residues and 876 water molecules. The apo-structure was refined to an *R*-factor of 15% and a  $R_{\text{free}}$  value of 17%. The root mean square differences between the monomers are  $\sim$ 0.2 Å. The complex structure with NADH and product was determined by soaking the crystals with a substrate, D-galacturonic acid, and a cofactor, NAD<sup>+</sup>. The resolution of the structure is 2.1 Å, the *R*-factor is 15.7%, and the  $R_{\rm free}$  value is 18.9%. The Y136A mutant complexed with NAD<sup>+</sup> was refined to 1.9 Å with an *R*-factor of 16.1% and an  $R_{\rm free}$  value of 18.6%. The root mean square differences between the molecules and models are ~0.2 Å.

Overall Structure—The AtUdh monomer is a single-domain protein consisting of seven  $\beta$ -strands and seven  $\alpha$ -helices. The structure of AtUdh contains a Rossmann fold motif with a parallel seven-stranded  $\beta$ -sheet ( $\beta 1-\beta 7$ ) surrounded by five  $\alpha$ helices ( $\alpha 2$ ,  $\alpha 3$ , and  $\alpha 4$  on one side and  $\alpha 1$  and  $\alpha 6$  on the other side). The cofactor is bound in a crevice at the C-terminal side of the  $\beta$ -sheet between  $\beta 1$  and  $\beta 4$ , in which the strand order is reversed. Two shorter  $\alpha$ -helices ( $\alpha 5$  and  $\alpha 7$ ) are located away from the main domain body and constitute parts of the substrate-binding cleft. The secondary structure elements of AtUdh are shown in Fig. 1*A*. Analysis of the crystal structure of AtUdh with the PISA server (17) suggests that the enzyme forms a hexamer with a total interface area of ~7450 Å<sup>2</sup>. Two monomers form a tightly packed dimer (interface area of 1400 Å<sup>2</sup>), and there is a 2-fold symmetry axis between these monomers. Helices  $\alpha$ 3 and  $\alpha$ 4 from both monomers are packed together to form a four-helical bundle (Fig. 1*B*). Three of these dimers are then more loosely packed together around a 3-fold symmetry axis, perpendicular to the symmetry axes between the tight dimers (Fig. 1, *B* and *C*). This packing results in a total of six monomer-monomer interfaces of 540 Å<sup>2</sup>. AtUdh was also found to be a hexamer in solution, as analyzed by size-exclusion chromatography and dynamic light scattering (data not shown).

*Free Cysteines*—AtUdh contains six free cysteine residues, which do not form disulfide bridges, typical for a cytosolic enzyme. Cys-65, Cys-166, and Cys-200 are embedded in the hydrophobic environment in the protein interior. The side chain of Cys-141 is on the protein surface but located on the monomer-monomer interface. Cys-47 and Cys-50, located in the  $\beta$ 3-strand, extend to the protein outer surface and may be theoretically capable of making intermolecular disulfide bridges in an oxidative environment.

Nucleotide-binding Site—In the electron density maps of the ternary complex of AtUdh, clear electron density was observed at the expected dinucleotide-binding site, which allowed an unambiguous fit of NADH with the electron density (Fig. 1*D*). All of the residues and solvent molecules that directly interact with NADH were identified (Fig. 2*B*). The bound NADH is located at the C-terminal edge of the  $\beta$ -sheet in an extended conformation. The adenine ring is positioned in an *anti*-conformation, the nicotinamide ring moiety is in a *syn*-conformation, and both riboses of the NADH adopt an C<sub>2</sub>-endo-conformation.



FIGURE 1. *A*, overall structure of the AtUdh monomer. *B*, hexameric structure of AtUdh. Two AtUdh units form a tight dimer (*purple* and *red*). A complete hexamer contains two additional dimers (*gray*). *C*, AtUdh hexamer as in *B* but rotated by 90°. *D*, electron density map around NADH and the product D-galactaro-1,5-lactone. *E*, electron density map around NAD<sup>+</sup> in the Y136A structure. The electron density omit maps ( $F_o - F_c$ ) are contoured at  $2\sigma$ .

The adenine ring of NADH interacts with the enzyme via hydrophobic interactions with Leu-35 and Leu-52 and a hydrogen bond between the adenine amino group and the Asp-51 carboxyl group. The 2'- and 3'-hydroxyl groups of the adenine ribose form hydrogen bonds with Asp-34. In addition, there is a hydrogen bond between the 2'-hydroxyl group and Ser-36. The presence of Asp-34 explains especially well why AtUdh does not accept NADP<sup>+</sup> as a cofactor, as the negatively charged phosphate at the 2'-position would repel a negatively charged carboxyl group of Asp-34. The pyrophosphate moiety is hydrogen-bonded to the main-chain atoms of Gln-14 and Leu-15 and to the side chain of Ser-75. The 3'-hydroxyl group of the nicotinamide ribose is hydrogen-bonded to Lys-140, and the 2'-hydroxyl group forms a hydrogen bond with Tyr-136. The nicotinamide ring has hydrophobic interactions with Ile-163, and the oxygen of the amide group forms a hydrogen bond with the main-chain nitrogen of Cys-166.

Substrate Binding—The refinement of the AtUdh apo-structure showed in the active site the electron density of a sulfate ion, which was used in crystallization (Fig. 2A). The oxygen atoms of the sulfate make hydrogen bonds with the side chains of Asn-112, His-113, Ser-165, and Arg-174. The sulfate was shown to inhibit AtUdh with an IC<sub>50</sub> of ~40 mM. Consequently, new crystallization conditions with ammonium phosphate were used instead.

The soaking of AtUdh crystals with NAD<sup>+</sup> and D-galacturonic acid resulted in a crystal structure that showed an electron density ring in the active site close to the nicotinamide ring of the NADH. This density was interpreted as the reaction product D-galactaro-1,5-lactone (Fig. 1*D*). The lactone ring stacks with the nicotinamide ring. The acid group of the lactone occupies the same position as the sulfate ion in the AtUdh apostructure, and it is hydrogen-bonded to the side chain of Arg-

174. The O1 carbonyl oxygen of the lactone is hydrogenbonded to Tyr-136 and Ser-111, and the O2 hydroxyl is bonded to the main-chain oxygen of Ser-75. The distance between the reactive C1 of the carbohydrate and nicotinamide C4 is 3.4 Å. As a result of this, the two molecules are very well placed for the hydride transfer.

Active-site Mutation Y136A-A putative proton acceptor, Tyr-136 was substituted with alanine to trap the substrate or a reaction intermediate in the crystal in the presence of NAD<sup>+</sup> and the substrate D-galacturonic acid. The activity of the purified Y136A mutant on D-galacturonic acid was  $\sim 0.1\%$  of the activity of wild-type AtUdh. The consequences of the mutation were also clearly visible in the electron density map. Surprisingly, there was no clear electron density for the substrate carbohydrate or for the nicotinamide residue of the NAD<sup>+</sup> cofactor. However, electron density was visible for the adenine and both phosphates of NAD $^+$  (Fig. 1*E*). The nicotinamide residue probably had multiple conformations because the electron density continues in several directions. Comparison of the mutant structure with the apo-structure and the ternary structure revealed an  $\sim$ 1.5-Å shift in the positions of Ile-74 and Ser-75 toward the nicotinamide ribosyl ring of NAD<sup>+</sup>. This may have restricted the binding of the nicotinamide end of the nucleotide and reduced enzymatic activity (Fig. 2D).

*Reaction Product Analysis*—Negative-ion electrospray ionization MS was used to analyze end product(s) of the oxidation reaction (supplemental Fig. S1). The mass spectrum of the starting compound, D-galacturonic acid (194 Da), showed an intense peak at m/z 193, corresponding to the  $[M - H]^-$  ion. After hydrogen/deuterium exchange, the peak was observed at m/z 197 instead (data not shown), consistent with the exchange of four hydrogens (accounting for four OH groups). The collision-induced dissociation MS/MS spectrum of D-galacturonic







FIGURE 2. A–C, active-site residues and ligands in apo-AtUdh, in the NADH and product ternary complex, and in the Y136A mutant structure, respectively. D, all three structures superimposed. NADH is in green, the product in cyan, and sulfate in yellow. E, superimposition of AtUdh (in purple) and S. venezuelae DesIV (in gray) showing the binding of NADH and product for AtUdh (in cyan) and substrate for DesIV (gray). F, binding of the reaction product D-galactaro-1,5-lactone showing the key distances (in Å) between the ligand (in cyan), cofactor (in green), and active-site residues (in purple).

acid showed a plethora of fragments. Two consecutive water losses (ions at m/z 175 and 157) were observed with the  $[M - H]^-$  ion. Alternatively, decarboxylation (elimination of CO<sub>2</sub>) was observed (m/z 149), which is highly characteristic for carboxylic acid anions, followed by two water losses (m/z 131 and 113) or loss of H<sub>2</sub>CO (m/z 119) or CH<sub>2</sub>(OH)CHO (m/z 89).

The mass spectrum of the reaction product(s) showed an intense peak at m/z 191 as well as a few other peaks that were identified as the impurities of the enzyme solution (supplemental Fig. S1, marked with asterisks). After hydrogen/deuterium exchange, the peak was observed at m/z 194 instead. These results are consistent with the formation of D-galactarolactone (192 Da), having three OH groups in the structure. The MS/MS spectrum of the reaction product showed a very intense peak at m/z 85 and less abundant fragment ions at m/z 173, 147, 129, and 111. This can be explained by decarboxylation (m/z 147), followed by a water loss (m/z 129) and a second decarboxylation (m/z 85). Inspection of the putative ion structures for the deprotonated D-galacturonic acid indicates that the fragmentation proceeds without ring opening (forming a stable cyclic carbanion structure, from which water eliminations can occur). In contrast, for the deprotonated D-galactarolactone (five-membered ring), the first decarboxylation results in the immediate ring opening, followed by water elimination and a second decarboxylation. This results in a fully conjugated, resonancestabilized ion at m/z 85. This is in line with the formation of a 1,4-lactone ring. In the case of a 1,5-lactone (six-membered ring), the first decarboxylation would result in the carbanion structure, similar to the ion at m/z 149, from which the ring opening cannot directly occur without rearrangement (*e.g.* hydride shift). Given the high abundance of the ion at m/z 85, it is evident that the more stable reaction product is D-galactaro-1,4-lactone. However, the initial reaction product is 1,5-lactone, as evidenced by crystallographic results. No other reaction products were detected.

## DISCUSSION

Substrate Specificity—The ternary crystal structure clearly suggests that the binding of the NAD<sup>+</sup> cofactor is a prerequisite for substrate binding because it participates in the formation of a ligand-binding site (Fig. 2*F*). The structure also shows that AtUdh is able to bind substrates in a pyranose form, *i.e.* as a cyclic six-membered ring. The specific activity of AtUdh has been studied recently by Yoon *et al.* (7) and more extensively by Boer *et al.* (6) These results are in good agreement with the crystal structures. AtUdh accepts both D-glucuronic acid and D-galacturonic acid as substrates. These sugar acids have a difference in the position of O4 hydroxyl group. According to the complex structure, the axial O4 hydroxyl group of D-galacturonic acid is toward a solvent and does not form any hydrogen



bonds, and there would be space for the O4 hydroxyl group in an equatorial position (Fig. 2*F*). D-Galactose, D-glucose, D-galactonic acid, and D-gluconic acid are not substrates for AtUdh (6). All of these compounds lack the carboxyl group at C6, which suggests that interaction of the carboxyl group with Arg-174 is essential for substrate binding.

Structure Comparison with Other SDRs—The SDR family is one of the largest enzyme families (18). However, there are only a few distantly related structures in the Protein Data Bank. Only dTDP-glucose 4,5-dehydratase (DesIV) from Streptomyces venezuelae shows some amino acid sequence identity (18%) to AtUdh (19). DesIV catalyzes a much more complicated reaction by oxidizing the 4'-hydroxyl group and by removing the 6'-hydroxyl group of the dUTP-glucose substrate. DesIV is a 55-residue larger protein than AtUdh. The NAD<sup>+</sup>-binding section is similar, but there are large differences in the structure, especially in the C-terminal region. The wild-type DesIV complex structure with NAD<sup>+</sup> and substrate does not show the electron density for glucose, whereas the inactive double mutant (D128N/E129Q) does. Superimposition of the cofactor- and substrate-binding sites of AtUdh and DesIV is shown in Fig. 2E. Despite large differences in the overall protein structures, especially in the loops, the binding modes for the cofactor and carbohydrates resemble each other.

*Mechanism*—The reaction catalyzed by AtUdh has been studied by NMR (6). The  $\beta$ -anomeric form of D-galacturonic acid was first consumed faster than the  $\alpha$ -anomer. This is explained nicely by the ternary complex structure, which would suggest that the O1 hydroxyl group of  $\alpha$ -D-galacturonic acid would clash with the nicotinamide ring of NAD<sup>+</sup>. Consequently, AtUdh would accept only  $\beta$ -D-galacturonic acid as a substrate. This is further formed in solution from  $\alpha$ -D-galacturonic acid by mutarotation.

NMR analysis also suggested that the reaction product would be D-galactaro-1,4-lactone (five-membered ring) and not D-galactaro-1,5-lactone (six-membered ring), as observed in the ternary complex structure. There was also a small amount of *meso*-galactarate in solution. This is in line with the observations made by electrospray ionization MS in this study. It is reasonable to assume that the reaction product D-galactaro-1,5-lactone rearranges non-enzymatically to D-galactaro-1,4-lactone, possibly via an open-chain *meso*-galactaric acid form (Fig. 3). Similar non-enzymatic 1,4-lactone formation has been observed previously for D-galactose dehydrogenases (20). Because the rearrangement is an equilibrium reaction, a very small amount of D-galactaro-1,5-lactone exists in solution and explains why this form was observed in the ternary complex crystal structure.

The catalytic mechanism of the dehydrogenase activity of the SDR family enzymes has been extensively discussed in light of structural, enzymological, and mutational data. It has been suggested that there would be a catalytic triad (Ser, Tyr, and Lys) or even a catalytic tetrad (Ser, Tyr, Leu, and Asn) and a proton relay system for SDRs (21). A classical proposed reaction mechanism for this enzyme family includes a transition state in which several bonds form and break at the same time (22). However, to describe the reaction as a "single-barrier" process might be an oversimplification. Rather, it could be considered



FIGURE 3. **Proposed reaction mechanism for AtUdh.** The locations of steps in water solution and in enzyme are indicated. In solution, the substrate D-galacturonic acid exists mainly in the equilibrium of  $\alpha$ - and  $\beta$ -pyranosic forms of the D-galacturonic acid (6), but only the  $\beta$ -anomeric form binds to the active site of the enzyme. In the enzymatic reaction, the removal of hydride by NAD<sup>+</sup> creates a resonance-stabilized cationic intermediate. The subsequent removal of a proton by Tyr-136 creates the reaction product D-galactaro-1,5-lactone, which is rearranged to the more stable D-galactaro-1,4-lactone in solution.

as a non-concerted or a stepwise reaction. Therefore, it is important to consider which is the first step. Proton donation from the O1 hydroxyl group to the catalytic tyrosine would create a negatively charged oxyanion, which would have high energy (verified normally by high  $pK_a$  values of hydroxyl groups). This kind of initiation has been proposed recently by Yasutake *et al.* (23). On the other hand, the hydride shift from C1 to NAD<sup>+</sup> would lead to the resonance-stabilized cationic intermediate. This intermediate would eagerly donate a proton to the neighboring group, in this case to Tyr-136, which in AtUdh is an unusually short distance (2.5 Å) from the carbonyl oxygen of the product (Fig. 2*F*). AtUdh could efficiently cata-





# Crystal Structure of Uronate Dehydrogenase

lyze the reaction containing a cationic intermediate through the nicotinamide ring, which packs against the substrate (Fig. 2F).

The catalytic triad or tetrad hypothesis emphasizes the role of four residues in proton transfer (21, 22). They all are present in AtUdh (Asn-87, Ser-111, Tyr-136, and Lys-140). Ser-111, and Tyr-136 are evidently essential residues in catalysis. Based on modeling studies, a lysine residue has been suggested to be part of a proton relay system from the phenolic oxygen of tyrosine to the 2'-hydroxyl group of ribosyl to this lysine (22). However, in AtUdh, Lys-140 is not hydrogen-bonded to the 2'-hydroxyl group but to the 3'-hydroxyl group of ribosyl (Fig. 2*B*). Also, in the DesIV structure, the corresponding lysine is hydrogen-bonded to the 3'-hydroxyl group (Fig. 2*E*). Therefore, the major function of lysine may be participation in NAD<sup>+</sup> binding, and the reason for conservation of asparagine in SDR structures may be mainly structural. If the first step in the catalysis of AtUdh is the hydride shift to NAD<sup>+</sup> and formation of a cationic intermediate, the participation of residues in proton abstraction might not be so critical. This might be reflected in the response in enzymatic activity to changes in pH. AtUdh has a high activity at pH 5-8, and the activity falls to 70% at pH 10 (7). If the proton abstraction from the substrate would be the key event, we could expect higher activity at high pH, which is not the case.

Overall, it is difficult to specify the role of individual residues because they may have several indistinguishable roles in protein structure, ligand binding, and catalysis. The Y136A mutant structure shows how unpredictable the consequences of one mutation can be. We tried to mutate the key catalytic residue, but this resulted in unexpected structural changes that affected cofactor binding.

Acknowledgments—We thank Armi Boman, Arja Kiema, and Outi Liehunen (VTT Technical Research Centre of Finland) and Ritva Romppanen (University of Eastern Finland) for technical assistance.

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Crystal Structure and Folding: Dehydrogenase from Agrobacterium tumefaciens



Tarja Parkkinen, Harry Boer, Janne Jänis, Martina Andberg, Merja Penttilä, Anu Koivula and Juha Rouvinen J. Biol. Chem. 2011, 286:27294-27300. doi: 10.1074/jbc.M111.254854 originally published online June 15, 2011

Access the most updated version of this article at doi: 10.1074/jbc.M111.254854

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