

Covalent Adduct Formation with a Mutated Enzyme: Evidence for a Thioester Intermediate in the Reaction Catalyzed by UDP-Glucose Dehydrogenase

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The enzyme UDP-glucose dehydrogenase catalyzes the 2-fold oxidation of UDP-glucose to UDP-glucuronic acid in an NAD⁺-dependent process.¹ In certain strains of pathogenic bacteria, such as group A streptococci² or *Streptococcus pneumoniae* Type 3,³ UDP-glucuronic acid is used in the biosynthesis of a polysaccharide capsule that serves to protect the bacteria from the host's defense mechanisms. The capsule is known to be a necessary requirement for virulence since acapsular, mutant strains are rendered avirulent.^{4–7}

The mechanism employed by the enzyme from *Streptococcus pyogenes* is thought to involve the initial oxidation of the C-6 alcohol to give an enzyme bound aldehyde intermediate **1** and NADH (Figure 1).^{8,9} This aldehyde is not released into solution and therefore has not been detected in the course of the normal enzyme reaction. It has, however, been independently synthesized and shown to be kinetically competent to serve as an intermediate.⁸ The second oxidation is thought to proceed via a hydride transfer from a thiohemiacetal intermediate to NAD⁺, generating NADH and a covalently bound thioester intermediate **2** (Figure 1, X = S). Hydrolysis of the thioester completes the reaction in a manner analogous to the well characterized enzyme, glyceraldehyde 3-phosphate dehydrogenase and to several other aldehyde dehydrogenases.^{10–13} Indirect evidence supporting the thioester intermediate is found in the observation that UDP-chloroacetyl irreversibly inactivates the enzyme via alkylation of an active site cysteine residue.⁹ In addition, all of the known sequences for this enzyme contain a conserved cysteine,^{2,7,14–16} and in the case

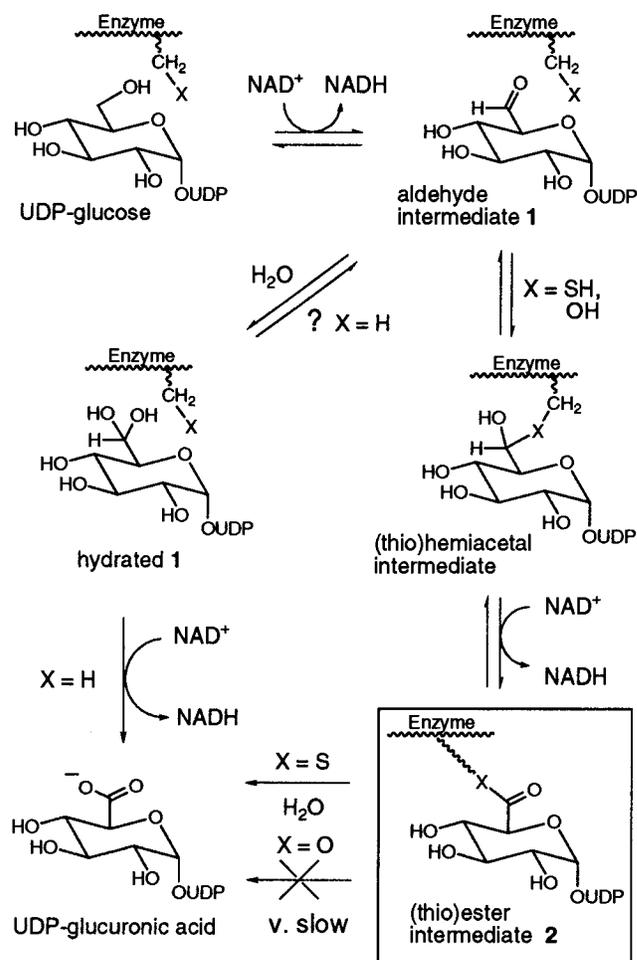


Figure 1.

of the beef liver enzyme, this cysteine has been implicated as an active site residue.^{14,17,18} In this Communication we describe the use of site directed mutagenesis to probe the role of Cys260 in the reaction catalyzed by the enzyme from *Streptococcus pyogenes*. We have found that incubation of the Cys260Ser mutant with UDP-glucose and NAD⁺ results in the formation of a covalent adduct whose mass and chemical reactivity are consistent with the acylenzyme **2** (Figure 1, X = O).

The Cys260Ala and Cys260Ser mutants¹⁹ were expressed and purified using an analogous procedure to that employed for the wild-type enzyme⁹ and were shown to have the expected masses by electrospray ionization mass spectrometry (ESI MS). When assayed under standard conditions, both mutant proteins showed less than 0.01% of the activity present in the wild-type enzyme, consistent with the notion that cysteine 260 is important for catalysis.⁹ Both the mutant and wild-type enzymes were then incubated for 1 h in the presence of 3 mM NAD⁺ and 1 mM of either UDP-glucose or UDP-glucosyl-hexodialdose **1**, and the resulting proteins were directly analyzed by ESI MS under

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(19) The Cys260Ala mutant was generated from plasmid pGAC400 using the pAlter site directed mutagenesis kit from Promega. Mutant Cys260Ser was produced using an adaptation of enzymatic PCR (Hughes, M. J. G.; Andrews D. W. *BioTechniques* **1996**, *20*, 188–196) using plasmid pGAC147. All mutations and constructs were confirmed by sequencing the entire gene.

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denaturing conditions.²⁰ In the case of the wild type enzyme and the Cys260Ala mutant, only the expected masses of the unlabeled proteins were observed. In the case of the Cys260Ser mutant however, 90% of the protein had a mass that was increased by an amount corresponding to the covalent attachment of one molecule of UDP-glucuronic acid, less one molecule of water (observed mass = 46 035 ± 6; unlabeled protein mass = 45 468; UDP-glucuronic acid mass = 580). Dialysis of the mutant-adduct for 24 h against pH 8.7, substrate-free, triethanolamine-HCl buffer at 5 °C resulted in the loss of <10% of the adduct (as analyzed by ESI MS), indicating that the attachment was kinetically stable in the active site of the folded protein.

To investigate whether the adduct was attached to Ser260, the labeled mutant was subjected to peptic digestion, and the resulting peptides were analyzed for the presence of an adduct. The technique of "neutral loss" tandem mass spectroscopy was used to identify labeled peptides as they eluted from a reversed-phase HPLC column.²¹ In this technique the mass spectrometer is used as a detector, and only peptides that lose a predetermined mass upon collision with argon are observed. Two peptides losing the mass of the UDP-sugar fragment (563 g/mol, lost due to cleavage of the bond between the carbonyl carbon and the serine oxygen) were detected and could be purified in microgram quantities.²² The masses of both intact peptides were consistent with expected peptic peptides containing the adduct at the site of the mutation: peptide A = GYGGYXLPKDKTQLL (observed mass for neutral peptide = 2201 ± 1), peptide B = GYGGYXLPKDKTKQ (observed mass for neutral peptide = 1974 ± 1) where X is the acylated Ser260. Identical results were obtained regardless of whether UDP-glucose or **1** were employed. The mass of peptide A was also determined at higher resolution using FT-ICR MS and gave data consistent with the molecular formula C₉₀H₁₃₈N₂₀O₄₀P₂ (neutral peptide): calcd mass = 2200.89; observed mass = 2200.91 ± 0.06.²³ To confirm the identity of peptide A, N-terminal sequencing by the Edman degradation method was performed, and the first five residues GYGGY were conclusively identified.²⁴

The peptide-adduct A was quite stable under mildly acidic or neutral conditions; however, a 15 min incubation at pH 10.2 and 40 °C resulted in loss of the adduct and produced the unmodified peptide as analyzed by ESI MS. If the pH of a solution of the peptide-adduct was adjusted to 10.2 and then immediately neutralized, negligible loss of the adduct was observed. This reactivity toward base-catalyzed hydrolysis is consistent with that expected for an ester linkage.²⁵

These results are interpreted as supporting the involvement of a thioester intermediate in the mechanism employed by UDP-glucose dehydrogenase. Conservative mutations at Cys260 caused

a dramatic loss of activity indicating that this residue is involved in catalysis. In the case of the serine mutant, prolonged incubations resulted in the formation of the corresponding ester intermediate (**2**, X = O).²⁶ This mutant can slowly catalyze both oxidation steps, but hydrolysis of the unnatural ester linkage is extremely slow, and therefore the adduct accumulates.²⁷ This is reminiscent of the enzyme, hydroxy-3-methylglutaryl-CoA reductase, which is capable of converting (*R*)-mevalonate and coenzyme A to the thioester, (*S*)-hydroxy-3-methylglutaryl-CoA, via a four-electron oxidation without hydrolysis.²⁸

A surprising observation was that the aldehyde **1** was readily oxidized by the alanine mutant ($k_{\text{cat}} = 0.19 \text{ s}^{-1}$, $K_M = 0.26 \text{ mM}$) in the presence of NAD⁺ (whereas UDP-glucose was not), and the catalytic constant was within an order of magnitude of that observed with the wild-type enzyme ($k_{\text{cat}} = 1.2 \text{ s}^{-1}$, $K_M = 0.014 \text{ mM}$). It appears that this mutant is able to catalyze the second step of the oxidation somewhat efficiently, even without a catalytic nucleophile. This is presumably occurring through the hydrated form of the aldehyde that resembles the thiohemiacetal intermediate and is able to bind in an analogous fashion (Figure 1).^{29,30} Previous studies on a sample of the bovine liver enzyme in which the active site thiol was cyanide-derivatized also showed that the modified protein retained a limited ability to catalyze the second oxidation step.¹⁸ The fact that the alanine mutant does not appreciably catalyze the oxidation of UDP-glucose could be explained by invoking a dual role for the Cys 260 thiol(ate); perhaps in the first oxidation step it assists in the deprotonation of the C-6 hydroxyl and in the second it is employed in thioester formation. Alternatively, it may be that the aldehyde **1** is tightly bound in the mutant active site, and there is no mechanism by which it can be hydrated and proceed forward in the second oxidation step.

In summary, the observation of adduct formation in the case of the Cys260Ser mutant leads us to conclude that the wild type enzyme operates via covalent catalysis using cysteine 260. The observation that the Cys260Ala mutant readily oxidizes **1**, however, indicates that in the absence of an active site nucleophile, the dehydrogenase is capable of catalyzing the second oxidation step without the involvement of covalent catalysis.

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(20) The incubations were carried out in 50 mM triethanolamine-HCl buffer, pH 8.7, at 30 °C. Samples were passed through a reversed-phase C18-silica gel HPLC column with an eluent of 0.05% trifluoroacetic acid in 1:3 CH₃-CN:H₂O before mass analysis by electrospray ionization mass spectrometry (ESI MS). The extended incubation time was necessary for complete adduct formation.

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(22) The technique relies on the loss of a neutral species, indicating that the pyrophosphate linkage was in a protonated state during the cleavage. It should also be noted that in the case of the glycosidase studies (see ref 21), the ester was formed between a glutamate side chain and an anomeric hydroxyl, and a distinctly different cleavage pattern was observed. The bond that broke in those studies was between the anomeric carbon of the carbohydrate and the oxygen of the carboxyl group.

(23) Fourier transform ion cyclotron resonance mass spectra (FT-ICR MS) were obtained by Stone D.-H. Shi at the National High Magnetic Field Laboratory, Tallahassee, FL using mellitin for external calibration. The value reported in the text was obtained from treatment of the mutant with NAD⁺ and UDP-glucose; treatment with NAD⁺ and **1** gave similar results (observed mass = 2200.88 ± 0.06).

(24) The quality of the sequencing data was insufficient to identify a thiohydantoin derivative for the sixth residue (Cys260). In any event, it is unclear whether the ester linkage would survive six rounds of Edman degradation analysis.

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(26) A cysteine-to-serine mutant of the analogous rat liver mitochondrial aldehyde dehydrogenase also gave an enzyme with very low activity, yet formation of the hemiacetal was proposed to be the rate determining step in that case (ref 12).

(27) Initial efforts have been made to observe the stoichiometric burst of NADH produced during the formation of the adduct by following the increase in A₃₄₀. Using NAD⁺ and **1**, a burst (over about 2 min) of approximately 0.7 equiv of NADH (per enzyme subunit) was detected. Using NAD⁺ and UDP-glucose, a very slow burst (over about 40 min) of approximately 0.7 equiv of NADH was seen. Both bursts were followed by an extremely slow, yet similar, rate of production of NADH that is presumably due to turnover of the common adduct via hydrolysis (half-life = 90 min). At this point it is unclear why the size of the burst was the same starting from either alcohol or aldehyde. It is clear from FT-ICR MS that "peptide A" formed from either source is at the same net oxidation state. It may be that a "hidden" hydride acceptor is responsible for the first oxidation and is only regenerated by NAD⁺ following hydrolysis of the (thio)ester intermediate. This is currently being investigated using [6-³H]UDP-glucose.

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(29) Compound **1** is known to exist primarily in the hydrated form when free in solution (see ref 8).

(30) A similar observation has been made with a cysteine-to-alanine mutant of glyceraldehyde-3-phosphate dehydrogenase; however, the reduction in k_{cat} was more dramatic, and the enzyme was converted from a phosphorylating dehydrogenase into a nonphosphorylating dehydrogenase (see ref 10).