UDP-glucuronic acid is used by many pathogenic bacteria in the construction of an antiphagocytic capsule that is required for virulence. The enzyme UDP-glucose dehydrogenase catalyzes the NAD⁺-dependent 2-fold oxidation of UDP-glucose and provides a source of the acid. In the present study the recombinant dehydrogenase from group A streptococci has been purified and found to be active as a monomer. The enzyme contains no chromophoric cofactors, and its activity is unaffected by the presence of EDTA or carbonyl-trapping reagents. Initial velocity and product inhibition kinetic patterns are consistent with a bi-uni-bi ping-pong mechanism in which UDP-glucose is bound first and UDP-glucuronate is released last. UDP-xylose was found to be a competitive inhibitor ($K_i = 2.7 \mu M$) of the enzyme. The enzyme is irreversibly inactivated by uridine 5’-diphosphate-chloroacetol due to the alkylation of an active site cysteine thiol. The apparent second order rate constant for the inhibition ($k_{i}/K_i$) was found to be $2 \times 10^3 \text{ mM}^{-1} \text{ min}^{-1}$. Incubation with the truncated compound, chloroacetol phosphate, resulted in no detectable inactivation when tested under comparable conditions. This supports the notion that uridine 5’-diphosphate-chloroacetol is bound in the place of UDP-glucose and is not simply acting as a nonspecific alkylating agent.

The enzyme UDP-glucose dehydrogenase (UDPGDH) catalyzes the NAD⁺-dependent oxidation of UDP-glucose to UDP-glucuronic acid (Fig. 1). It belongs to a small group of dehydrogenases that are able to carry out the 2-fold oxidation of an alcohol to an acid without the release of an aldehyde intermediate (1). Much of the work to date has focused on the properties and mechanism of the beef liver enzyme, and relatively little is known about the enzyme purified from bacterial sources (2–4). In many strains of bacteria that act as human pathogens, UDPGDH provides the UDP-glucuronic acid required for the construction of an antiphagocytic capsular polysaccharide. It is well established that the formation of the capsule is required for virulence (5, 6), and it is thought that the capsule enables the bacteria to evade the host’s immune system (7, 8). Group A and C streptococci are mammalian pathogens that use UDPGDH in the synthesis of a capsule composed of hyaluronic acid (a polysaccharide consisting of alternating glucuronic acid and N-acetylglucosamine residues) (9, 10). Many of the known strains of Streptococcus pneumoniae also use UDP-glucuronic acid in the construction of their polysaccharide capsule (11), and it has recently been shown that UDPGDH is required for capsule production in S. pneumoniae (type 3) (12). The encapsulated Escherichia coli K5 is also known to use the enzyme for a similar purpose (2, 13). The hasB gene that encodes for UDPGDH in group A streptococci (Streptococcus pyogenes) has been cloned and overexpressed in Escherichia coli (14). Its gene product shares 57% sequence identity and 74% sequence similarity with the S. pneumoniae enzyme (15, 16). These properties make the HasB UDPGDH an attractive target for further mechanistic studies and inhibitor design.

The mechanism used by the bovine liver dehydrogenase is thought to involve an initial oxidation of the C-6 hydroxyl of UDP-glucose to form an aldehyde intermediate and NADH (Fig. 1) (17–21). The aldehyde is tightly bound to the enzyme and is inaccessible to carbonyl-trapping reagents (17, 18). It has been suggested that the aldehyde is bound via an imine linkage with a lysine residue (19), however this is somewhat at odds with solvent oxygen isotope incorporation studies (20). The second oxidation is initiated by the addition of an active site thiol to the aldehyde to form a thiohemiacetal (21). A hydride transfer from the intermediate to NAD⁺ then takes place to produce an enzyme-bound thioester intermediate and a second molecule of NADH. In a final irreversible step the thioester is hydrolyzed to give the product UDP-glucuronic acid. The formation of a thioester intermediate in the second oxidation step is preceded by studies on glyceraldehyde-3-phosphate dehydrogenase and several other aldehyde dehydrogenases (22–25).

The existence of an active site thiol residue has been well documented in the case of UDP-glucose dehydrogenase from beef liver. Thiol modification studies using iodoacetate (26), iodoacetamide (26), 5-[[iodoacetamido]ethylamino]naphthalene-1-sulfonic acid (27), iodoacetamidofluorescein (28), 5,5'-dithiobis-(2-nitrobenzoate) (21, 29), and 6,6'-dithionoic-tinate (30) all indicate that a cysteine is required for catalysis. The observation that the cysteine is protected from modification by the addition of UDP-glucose or UDP-xylose suggests that it is located in the active site (26, 29). Proteolytic digestion and sequencing studies on the labeled enzyme have indicated that Cys-275 supplies the reactive thiol (28, 31). The use of relatively nonspecific thiol labeling reagents can, however, lead to misleading results in certain cases (see Ref. 24 and references therein). The enzyme histidinol dehydrogenase also catalyzes the NAD⁺-dependent 2-fold oxidation of an alcohol to an
Acid (without the release of aldehyde) and was generally thought to use a mechanism similar to that of UDP-glucose dehydrogenase (1). Treatment of the enzyme with 7-chloro-4-nitro-2,1,3-benzoxadiazole resulted in the labeling of an active site cysteine and the loss of enzyme activity (32). This seemed to indicate that Cys-116 supplied a thiol that participated in the second oxidation step. Subsequent mutagenesis experiments indicated, however, that this enzyme did not require a cysteine for catalysis and therefore does not follow the glyceraldehyde-3-phosphate dehydrogenase paradigm (33, 34).

A way in which one can maximize the chances that a labeling reagent will target a catalytically important group is to incorporate structural elements of the natural substrate into its design. In the case of UDP-glucose dehydrogenase the diphosphate nucleotide group provides a convenient handle that can be used to orient a reactive moiety within the active site. The reagent uridine 5′-diphosphate chloroacetol (UDC) was first synthesized by Flentke and Frey (35) and positions an electrophilic carbon atom three bond lengths away from the β-phosphate of a UDP group (Fig. 1, inset). It is therefore a useful tool in probing the active site of UDP sugar nucleotide-modifying enzymes.

In this article we report the purification and initial characterization of the recombinant HasB UDPGDH. The streptococcal enzyme is notably different from the mammalian enzyme in that it is a monomer (as opposed to a hexamer). The initial velocity and product inhibition patterns are shown to be consistent with a bi-unii-bi ping-pong mechanism (36) in which UDP-glucose is bound first and UDP-glucuronic acid is released last. We have also demonstrated that UDP is an active site-directed, irreversible inhibitor of the enzyme and covalently modifies a cysteine thiol. The apparent second order rate constant for the inhibition (k/K) was found to be 2 x 10^3 M^-1 min^-1. Incubation with the truncated compound, chloroacetol phosphate, resulted in no detectable inactivation when tested under comparable conditions and concentrations.

**Experimental Procedures**

Purification and Properties of UDPGDH—The hasB gene product was overexpressed in *E. coli* JM109 (DE3, pGAC147) as described elsewhere (14). The cells (1.9 g) were pelleted 3 h after induction with isopropyl-1-thio-β-D-galactosidase. They were resuspended in buffer A (5 ml; 50 mM Trien-HCl buffer, pH 8.7, containing glycerol (10%, v/v), dithiothreitol (2 mM), pepstatin (1 mg/ml), aprotinin (1 mg/ml), and phenylmethanesulfonyl fluoride (1.5 mM)). Cells were lysed by two passes through a French pressure cell at 20,000 p.s.i. Following ultracentrifugation at 30,000 rpm (60,000 x g) for 1 h, the resulting supernatant was loaded onto a column (15 ml) of diethylami-}

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**UDP-glucose Dehydrogenase from Group A Streptococci**

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noethylicelullose that had been pre-equilibrated with buffer A. The column was washed with buffer A (50 ml) and then eluted with buffer A containing 200 mM NaCl (50 ml). Active fractions were pooled and concentrated (to 6.7 ml) using Centriprep-10 concentrators and dialyzed against 1 liter of buffer B (50 mM Trien-HCl buffer, pH 8.7, containing glycerol (10%, v/v) and dithiothreitol (2 mM)) for 48 h. The resulting solution was frozen in liquid nitrogen and stored at -75°C. The protein solution was quickly thawed and applied (approximately 27 mg of total protein in each injection) to a Waters AP-1 Protein-Pak Q column (10 x 100 mm) that had been equilibrated with buffer B (at 22°C). The column was washed with buffer B (10 ml) and eluted with a linear gradient of NaCl (0 – 120 mM, over 35 min at 1.3 ml/min) in buffer B. The active fractions were pooled, frozen in liquid nitrogen, and stored at -75°C. Unless noted otherwise, all manipulations were performed at 4°C. Protein concentrations were determined by the method of Bradford (37), using bovine serum albumin as a standard. A unit of enzyme is defined as the amount of enzyme required to produce 2 μmol of NADH/min at 30°C.

The subunit molecular weight was determined on a Perkin-Elmer Sciex API300 electrospray mass spectrometer. The native molecular weight was estimated using gel filtration chromatography on a Protein-Pak 300SW column (waters). The column was run in potassium phosphate buffer (100 mM, pH 7.0) at a flow rate of 1 ml/min. Blue dextran (2,000 kDa) was used to mark the void elution time, and bovine serum albumin (66 kDa), ovalbumin (43 kDa), and cytochrome c (12.4 kDa) were used as molecular weight standards.

**UV Spectrum of the Dehydrogenase**—A sample of UDPGDH (0.4 ml of a 2.5 mg/ml solution) purified as described above was dialyzed twice for 24 h each time against potassium phosphate buffer (1 liter, 10 mM, pH 8.7, containing 2 mM dithiothreitol). To obtain a clean spectrum of the enzyme it was necessary to prevent the aerobic oxidation of dithiothreitol by carrying out the dialysis under an atmosphere of argon. The buffer was degassed and thoroughly sparged with argon prior to the addition of solid dithiothreitol. Following dialysis, particular solute was removed from the sample by centrifugation at 10,000 rpm for 15 min. A UV spectrum of the enzyme (final concentration, 1.15 mg/ml or 25 μM) was recorded at 30°C. A second spectrum was recorded following the addition of excess UDP-glucose (final concentration, 500 μM). A final spectrum was recorded following the addition of both UDP-glucose (500 μM) and 1 eq of NAD+ (25 μM).

**Enzyme Kinetics**—UDP-Glucose dehydrogenase activity was assayed using a modification of a previously described procedure (4). Rates were determined by following the reduction of NAD+ at 340 nm using a Varian Cary 3E UV-visible spectrophotometer. All assays were performed at 30°C in 50 mM Tris-HCl buffer, pH 8.7, containing 2 mM dithiothreitol (1 ml total volume). Initial velocities were measured during the first 40 s after initiation with UDPGDH (2.0 x 10^-4 units), and slopes were calculated using a least squares analysis with Cary 3 software version 3.0. NADH concentrations were determined using A_{440} with ε = 6,220 M^-1 cm^-1. The compounds NAD+, NADH, UDP-glucose, UDP-acid, and UDP-xylene were all purchased from Sigma.

**Inactivation Studies**—UDPGDH was prepared according to the procedure of Flentke and Frey (35), and chloroacetol phosphate was prepared as described by Hartman (38). The concentration of UDC was determined using A_{340} with ε = 8,900 M^-1 cm^-1 (35). Inactivation kinetics were measured in the absence of dithiothreitol to prevent the reaction of the inhibitor with added thiols. Inactivation incubations were therefore performed under an atmosphere of argon, and aliquots were removed by syringe. The buffer was degassed and sparged with argon prior to use. In the absence of inhibitor the enzyme was shown to lose negligible amounts of activity under these conditions. The inactivations were carried out at 30°C in 50 mM Tris-HCl buffer, pH 8.7, containing either 5.0 mM UDP-glucuronic acid or 75 μM UDP-glucose and the inhibitor (0.5 ml total volume). Timed aliquots (50 μl) were removed and diluted in the assay buffer (900 μl containing 500 μM UDP-glucose) prior to analysis. The initial velocities were obtained following inactivation with NAD+ (500 μM). First order rate constants describing the loss of enzyme activity were obtained using the program Grafit.

The sample used to determine the mass of the inhibited enzyme was prepared in the following manner. A sample of UDPGDH (1.2 mg/ml in the assay buffer) was incubated at 37°C for 30 min in the presence of UDP (2.0 mM). Any remaining inhibitor was quenched by the addition of excess dithiothreitol (20 mM) prior to analysis. To determine the nature of the tagged residue a sample of UDPGDH (2 mg/ml in 40 μl of the assay buffer) was inactivated by incubation at 30°C for 30 min in the presence of UDC (250 μM). A second sample was incubated under identical conditions in the absence of UDC. Both samples were denatured by the addition of saturated urea (40 μl pre-
pared in the assay buffer lacking diithiothreitol). Following 1 h of incubation at room temperature, iodoacetate was added to each sample (to a final concentration of 1 mM). The solutions were incubated in the dark for an additional 1 h and then quenched by the addition of excess diithiothreitol (to a final concentration of 5 mM) prior to mass spectral analysis.

RESULTS

Purification and Properties of UDPGDH—The dehydrogenase was purified to homogeneity (as judged by SDS-polyacrylamide gel electrophoresis) by ion exchange chromatography. The purification gave 35 mg of protein/500 ml of culture, which accounts for 73% of the activity in the crude extract (Table I). A specific activity of 1.5 units/mg was measured. The mass of the protein was 45,489 ± 4 Da (versus 45,487 Da calculated) as determined by electrospray mass spectrometry.

The enzyme was found to fairly stable when stored in the presence of 2 mM diithiothreitol. Incubation for 1.6 h at 30 °C (50 mM trien-HCl, pH 8.7) resulted in the loss of 40% of the activity. Similar incubations in the absence of any reduced thiol resulted in the loss of 75% of the activity. When stored at 5 °C (in the presence of dithiothreitol) the enzyme was found to be quite stable (greater than 90% of the activity remained after 24 h). The enzyme was also stable to repeated rapid freeze-thaw cycles when kept in a buffer containing 10% glycerol and diithiothreitol.

Gel filtration chromatography indicated that the enzyme has a mass of approximately 52 kDa when compared with known standards (Fig. 2). This indicates that the enzyme is active as a monomer.

Effect of Additives on the Enzymic Reaction—As mentioned previously, the activity of the dehydrogenase was dependent on the presence of reduced thiols such as diithiothreitol. The activity was essentially unaffected by the addition of 5 mM EDTA, indicating that exchangeable divalent cations were not required.

To investigate whether the putative aldehyde intermediate is released or is accessible to solvent, the stoichiometry of the reaction was determined in the presence of the aldehyde-trapping reagents semicarbazide and hydroxylamine. The ratio of NADH produced/UDP-glucose added was found to be constant (about 1.9:1) over a range of 0–50 mM of each of the additives.

UV Spectrum of the Purified Dehydrogenase—The UV spectrum of the dehydrogenase (Fig. 3, trace 1) did not show any significant absorptions past 300 nm that might be indicative of a bound chromophore such as NADH. The addition of excess UDP-glucose to the cuvette did not cause any changes in this area of the spectrum (Fig. 3, trace 2). This indicates that bound NADH is not present, since the equilibrium lies strongly in favor of NADH and UDP-glucuronic acid (17). When one equivalent of NADH was added to the same cuvette, absorbance due to the NADH chromophore was clearly observed (340 nm maximum; Fig. 3, trace 3). These studies suggest that only stoichiometric amounts, if any, of NADH or NADH are present in the enzyme sample.

Initial Velocity Kinetic Studies—The initial velocity kinetic patterns with UDP-glucose as the variable substrate are shown in Fig. 4, and those with NADH as the variable substrate are shown in Fig. 5. In both cases linear intersecting patterns are observed, indicating that the binding of UDP-glucose and the first NADH are reversibly connected (39). It also suggests that the binding of the first NADH and the second NADH are not reversibly connected (a reversible connection should result in nonlinear initial velocity patterns) (39). This indicates that a ping-pong mechanism is operative in which the release of the product is not present, since the equilibrium lies strongly in favor of NADH and UDP-glucuronic acid (17). When one equivalent of NADH was added to the same cuvette, absorbance due to the NADH chromophore was clearly observed (340 nm maximum; Fig. 3, trace 3). These studies suggest that only stoichiometric amounts, if any, of NADH or NADH are present in the enzyme sample.

Product Inhibition Studies—Product inhibition studies were performed by including changing fixed concentrations of either UDP-glucuronic acid or NADH in the assay buffer and varying the concentration of either UDP-glucose or NADH in the presence of saturating concentrations of the other substrate (200 mM UDP-glucose or 500 mM NADH). When UDP-glucuronic acid was the inhibitor and UDP-glucose was the variable substrate, a double reciprocal plot of the initial velocity data revealed a pattern consistent with competitive inhibition (Fig. 6). This indicates that the two species are...
interacting with the same enzyme form and is consistent with a kinetic mechanism in which UDP-glucose binds first and UDP-glucuronic acid is released last (39). A replot of the slopes against the UDP-glucuronic acid concentration (Fig. 6, right graph) gave a $K_i$ of 200 $\mu$M for UDP-glucuronic acid. The apparent small differences in $V_{max}$ seen in Fig. 6 could potentially be caused by weak substrate inhibition. When UDP-glucuronic acid was the inhibitor and NAD$^+$ was the variable substrate (with saturating levels of UDP-glucose), no inhibition was observed. This is entirely consistent with the observation of competitive inhibition between UDP-glucuronic acid and UDP-glucose. Since the two UDP sugars bind to the same form of the enzyme, saturation with UDP-glucose will prevent the binding of UDP-glucuronic acid.

When NADH was the inhibitor and UDP-glucose was the variable substrate (in the presence of saturating NAD$^+$), a parallel pattern of lines resulted (Fig. 7). This apparent uncompetitive inhibition is seen if the two species are interacting with different enzyme forms that are not reversibly connected (39). It is consistent with a mechanism in which one or both of the NAD$^+$ molecules bind to the enzyme after UDP-glucose binds and before the first NADH is released. When NADH was the inhibitor and NAD$^+$ was the variable substrate, an apparent noncompetitive pattern was observed (Fig. 8). This pattern indicates that at least one NADH molecule and one NAD$^+$ molecule interact with different enzyme forms, and the points of interaction are not separated by any effectively irreversible steps, such as the binding of UDP-glucose (when present at saturating levels) or the release of UDP-glucuronic acid (when absent from the solution).

**Inhibition by UDP-Xylose**—Inhibition by UDP-xylose was investigated by including changing fixed concentrations of UDP-xylose in the assay buffer and varying the concentration of UDP-glucose in the presence of saturating concentrations of NAD$^+$ (500 $\mu$M). A double reciprocal plot of the data showed a pattern consistent with competitive inhibition (Fig. 9). A replot of the slopes against the UDP-glucuronic acid concentration (Fig. 9, right graph) gave a $K_i$ of 2.7 $\mu$M for UDP-xylose.

**Irreversible Inhibition by UDC**—Incubation of UDPGDH with 0.20 mM solution of UDC resulted in the rapid inactivation of the enzyme. Dialysis of the inactivated enzyme against inhibitor-free buffer for 48 h did not result in the restoration of any measurable activity, indicating that the process is irreversible. An electrospray mass spectrum of the inactivated enzyme showed that the mass of the protein increased from 45,493 ± 4 to 45,958 ± 4 Da on inactivation. This is consistent with the covalent attachment of one molecule of inhibitor (molecular weight, 495 g/mol) to the enzyme with an accompanying loss of a chlorine atom. The presence of known competitive inhibitors
Assays were performed at 30 °C in 50 mM Tris-HCl, pH 8.7, containing 2 mM dithiothreitol and 200 μM UDP-glucose. The reactions were initiated by the addition of 0.02 units of UDPGDH. NADH concentrations are 0 μM (○), 25 μM (△), 45 μM (□), 65 μM (■), 87 μM (□), 110 μM (●), and 140 μM (○).

Figure 8. NADH inhibition pattern with NAD⁺ as variable substrate. Assays were performed at 30 °C in 50 mM Tris-HCl, pH 8.7, containing 2 mM dithiothreitol and 200 μM UDP-glucose. The reactions were initiated by the addition of 0.02 units of UDPGDH. NADH concentrations are 0 μM (○), 25 μM (△), 45 μM (□), 65 μM (■), 87 μM (□), 110 μM (●), and 140 μM (○).

Figure 9. UDP-Xylose (UDPX) inhibition pattern with UDP-glucose (UDPG) as variable substrate. A replot of the slopes against [UDP-xylose] is shown on the right graph. Assays were performed at 30 °C in 50 mM Tris-HCl, pH 8.7, containing 2 mM dithiothreitol and 500 μM NAD⁺. The reactions were initiated by the addition of 0.02 units of UDPGDH. UDP-xylose concentrations are 0 μM (○), 0.5 μM (●), 1.5 μM (□), 3.0 μM (■), 4.5 μM (△), 7.0 μM (▲), and 10 μM (○).

The rate constant for inactivation (kobs) will be lowered according to Equation 1:

$$k_{obs} = \frac{k[I]}{K \left(1 + \frac{[F]}{K_F} \right) + [I]}$$  \hspace{1cm} (Eq. 1)

where I is the irreversible inhibitor, and P is the known competitive inhibitor. In the presence of 5.1 mM UDP-glucuronic acid (Kᵢ, 200 μM), UDC was found to inactivate the dehydrogenase in a pseudo-first order fashion, as shown in Fig. 10. The rapid inhibition kinetics dictated that our measurements could only be made at UDC concentrations well below the value of Kᵢ; therefore, only the apparent second order rate constant k/Kᵢ could be obtained. A plot of kobs against the inhibitor concentration (Fig. 10, inset) gave the value of k/Kᵢ = 1.6 × 10⁷ M⁻¹ min⁻¹. To support the assumption of competitive binding made by Equation 1, the inactivation was also followed in the presence of 75 μM UDP-xylose (Kᵢ, 2.7 μM). A similar analysis (data not shown) gave a value of k/Kᵢ = 2.1 × 10⁷ M⁻¹ min⁻¹, which is in reasonable agreement with that obtained in the presence of UDP-glucuronic acid.

To demonstrate that UDC was acting as a specific irreversible inhibitor of the enzyme, as opposed to a relatively nonspecific alkylating agent, the inactivation by chloroacetol phosphate (38) was briefly investigated. Chloroacetol phosphate contains the chemically reactive α-haloketone moiety required for covalent modification to occur yet lacks the terminal UMP functionality that is likely important in active site binding. When the dehydrogenase was incubated in the presence of 17 μM UDC and 5 mM UDP-glucuronic acid, 50% of the activity was lost within 1 minute (see Fig. 10). Analogous incubations with 17 μM chloroacetol phosphate, however, showed no detectable inactivator-dependent loss of activity over a 10-min period (2–3% of the activity is lost even in the absence of inactivator). When the concentration of chloroacetol phosphate was increased to 2 mM (with 5 mM UDP-glucuronic acid) only 25% of the activity was lost over a 10-min incubation. Incubation with 2 mM chloroacetol phosphate in the absence of any competitive inhibitor resulted in inactivation with a half-life of approximately 1 min. These results show that the terminal UMP functionality is required for the potency of UDC and is consistent with a specific binding event occurring prior to alkylation.

The nature of the tagged residue was investigated in the following manner. A sample of the dehydrogenase was denatured in 5 M urea and then treated with excess iodoacetate. An electrospray mass spectrum showed that the mass of the protein increased from 45,493 ± 4 to 45,606 ± 4 Da, consistent with the attachment of two acetate units (expected difference, 116 daltons). A similar analysis using an enzyme previously inactivated by UDC showed that the mass of the protein-inhibitor adduct increased from 45,958 ± 4 to 46,009 ± 4 Da, consistent with the attachment of only 1 acetate unit (expected difference, 58 daltons). The enzyme contains only two cysteine residues, and in a denatured state both will be readily alky-
lated by iodoacetate (as indicated by the mass spectral data). The observation that prior inactivation with UDC results in the removal of one of the sites of acetate attachment strongly indicates that the inhibitor acts by alkylating an active site cysteine residue.

**DISCUSSION**

A well documented characteristic of the hexameric bovine liver UDP-glucose dehydrogenase is that it displays half-of-the-sites reactivity (26, 30, 40, 41). The enzyme binds only three molecules of UDP-glucose at saturation, and only three of its six active site cysteine thiols are readily accessible to alkylation by reagents such as iodoacetic acid. Studies on partially denatured samples of the beef liver enzyme led researchers to speculate that two active sites, one on each subunit, were involved in the overall reaction (40). They suggested that the first oxidation produces an aldehyde intermediate, which is covalently bound via an imine linkage with a lysine residue. The intermediate is then transferred to a cysteine thiol in the active site of an adjacent subunit where the second oxidation takes place. Resonance energy transfer experiments were found to be at odds with this notion, since they indicated that the six active sites of the hexamer were spatially remote (27). The observation made in this article that the streptococcal enzyme is monomeric is significant in that it rules out any mechanisms invoking the participation of more than one subunit. The sequence similarities (31% positional identity) between the streptococcal enzyme and the bovine liver enzyme suggest that they share a common ancestry and likely use a common mechanism (31).

The only other bacterial UDP-glucose dehydrogenase that has been purified is from *E. coli* (2–4). Many of the properties of the streptococcal enzyme are similar to that of the *E. coli* enzyme, with the notable exceptions that the *E. coli* enzyme is reported to be active as a dimer and that the UDP-glucuronic acid inhibition patterns display positive cooperativity. The observation that the dehydrogenase activity is dependent on the presence of reduced thiols and is unaffected by reagents such as iodoacetic acid. Studies on partially denatured samples of the beef liver enzyme led researchers to speculate that two active sites, one on each subunit, were involved in the overall reaction (40). They suggested that the first oxidation produces an aldehyde intermediate, which is covalently bound via an imine linkage with a lysine residue. The intermediate is then transferred to a cysteine thiol in the active site of an adjacent subunit where the second oxidation takes place. Resonance energy transfer experiments were found to be at odds with this notion, since they indicated that the six active sites of the hexamer were spatially remote (27). The observation made in this article that the streptococcal enzyme is monomeric is significant in that it rules out any mechanisms invoking the participation of more than one subunit. The sequence similarities (31% positional identity) between the streptococcal enzyme and the bovine liver enzyme suggest that they share a common ancestry and likely use a common mechanism (31).

The kinetic mechanism of the bovine liver UDP-glucose dehydrogenase; however, few details concerning the enzyme nucleophile is taking place. It should be noted that a related compound, α-bromoacetamidophenyl uridyl pyrophosphate, has been reported to be a potent inhibitor of UDP-glucose dehydrogenase; however, few details concerning the nature of the inhibition have been published (46, 47).

The observation that the inactivation by UDC is slowed by the addition of known competitive inhibitors indicates that the process is active site-directed. Furthermore, the agreement in the values of $k/K_i$ obtained in the presence of two different competitive inhibitors indicate that the initial binding of UDC is competitive with that of UDP-glucose. The assumption that the UDF group of UDC occupies the normal UDP binding site is supported by the observation that the truncated compound, chloroacetol phosphate, is a markedly less potent inhibitor. The electrophilic carbon of bound UDC should therefore rest in a position comparable with that of the aldehyde during the course of the normal reaction pathway. The iodoacetate-labeling experiments clearly indicate that one thiol has been modified during the inactivation process and support the notion that a cysteine is in the proper vicinity to participate in covalent catalysis. When the protein sequence of the streptococcal enzyme is compared with that of the other known UDFGDHs, Cys-260 is strictly conserved, whereas Cys-162 clearly is not (14–16, 31, 48). This observation, combined with the previous identification (28, 31) of the iodoacetate-labeled tryptic fragment from the bovine liver enzyme (containing the corresponding conserved residue Cys-275), strongly indicates that Cys-260 is the active site thiol. Further studies involving site-directed
mutagenesis and proteolytic digestion techniques will be undertaken to investigate this further.

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