Mechanistic Studies of the Biosynthesis of 3,6-Dideoxyhexoses in 
_Yersinia pseudotuberculosis_

PURIFICATION AND STEREOCHEMICAL ANALYSIS OF CDP-D-GLUCOSE OXIDOREDUCTASE*

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An NAD⁺-dependent CDP-D-glucose oxidoreductase which catalyzes the first step of the biosynthesis of CDP-ascarylose (CDP-3,6-dideoxy-L-arabino-hexose), converting CDP-D-glucose to CDP-4-keto-6-deoxy-D-glucose, was isolated from _Yersinia pseudotuberculosis_. A protocol consisting of DEAE-cellulose, Matrix Blue-A, hydroxyapatite, DEAE-Sephadex, Sephadex G-100, and NAD⁺-agarose column chromatography was used to purify this enzyme 6000-fold to homogeneity. This enzyme consists of two identical subunits, each with a molecular weight of 42,500. Using CDP-D-glucose as the substrate, the $K_m$ and $V_{max}$ of this catalytic event were determined to be 222 μM and 8.3 μmol min⁻¹, respectively. Unlike most other oxidoreductases of this class which have a tightly bound NAD⁺, this highly purified CDP-D-glucose oxidoreductase showed an absolute requirement of NAD⁺ for its activity. Using chemically synthesized (6S)- and (6R)-CDP-[4-²H₆,6-³H₁]glucose as substrates, a stereochemical analysis showed this enzymatic reaction involves an intramolecular hydroxyl group migration from C-4 to C-6, and the displacement of C-6 hydroxy group by the C-4 hydrogen occurs with inversion. Thus, despite the low cofactor affinity, this enzyme undergoes a mechanism consistent with that followed by other members of its type. Such a mechanistic and stereoconvergent pathway for all sugar oxidoreductases so far characterized suggests the presence of a common progenitor of this class of enzyme.

The 3,6-dideoxyhexoses are an important class of carbohydrates. They are found, with few exceptions (1), only in the lipopolysaccharide component of the cell wall of Gram-negative bacteria in which they constitute the nonreducing terminal groups of the O-antigen repeating units (2–4). Since the immunological specificities of Gram-negative species are mainly determined by the nonreducing sugar entities of these O-antigen repeating units, the 3,6-dideoxyhexoses have been shown to confer unique serological specificities in many immunological active lipopolysaccharides. Thus, they are commonly referred as immunodominant sugars and/or antigenic determinants in bacteria (5–9). Biosynthesis of these unusual sugars follows a complex pathway starting with an internal oxidation-reduction step mediated by an NAD⁺-dependent oxidoreductase. This enzyme catalyzes the transformation of a nucleotidyl diphosphohexose (1) to the corresponding 4-keto-6-deoxyhexose derivative (2) (10–12) which, upon further catalysis by a dehydrase and a reductase, is converted to a 3,6-dideoxyhexose as the final product (13–15). It has been shown that this oxidoreductase catalyzed reaction is a key step common to the biosynthesis of many unusual carbohydrates (4).

A number of this type of oxidoreductase have been found in nature. These include the TDP-D-glucose oxidoreductase from _Pseudomonas aeruginosa_ (16), _Escherichia coli_ (17–19), _Streptomyces rimosus_ (20), and _Saccharopolyspora erythraea_ (21); the CDP-D-glucose oxidoreductase from _Pasturella pseudotuberculosis_ (22) and several strains of _Salmonella_ sp. (23); the GDP-D-mannose oxidoreductase from _Aerobacter aerogenes_ (24), _E. coli_ (25), plants (26), and porcine thyroid (27). This class of enzymes is characteristic for an active-site bound nicotinamide coenzyme. As depicted in Scheme I (NDP represents a generic nucleoside diphosphate group), studies of the homogeneous TDP-D-glucose oxidoreductase from _E. coli_ had shown that this enzymatic reaction proceeds with an oxidation at C-4, a dehydration between C-5 and C-6, and a reduction at C-6 (10–12). Such a transformation is accompanied by an intramolecular hydrogen transfer from C-4 of the substrate to C-6 of the resulting 4-keto-6-deoxyhexose product (17, 28) with the enzyme-bound NAD⁺ serving as a hydride carrier. An examination of the biochemical reaction of the _E. coli_ enzyme (29) found that the displacement of the hydroxyl group at C-6 by the hydrogen from C-4 occurs with inversion, the dehydration from C-5 and C-6 is a syn elimination, and the internal hydrogen transfer to NAD⁺ is "anti"face-specific (30). The same mechanistic conclusions were also reached for the GDP-D-mannose oxidoreductase isolated from an unidentified soil bacterium (ATCC 19241) (31). Thus, enzymes of this type obtained from different sources may have distinct substrate specificity, but they all seem to share a common reaction pathway.

Despite the fact that the mechanistic similarity of enzymes within this class has been well established, studies of the oxidoreductases isolated from _S. erythaeras_ (21) and _P. pseudotuberculosis_ (22, 32) showed an absolute requirement of NAD⁺ for activity that is fundamentally distinct from most of their counterparts. Since the enzyme-bound nicotinamide cofactor in this type of enzyme is known to play a pivotal role in subunit association and its redox state is crucial in regu-
lating substrate binding and product releasing (33, 34), the observation of weak NAD\(^+\) binding in the *Pasturella* and *Saccharopolyspora* enzymes throws into doubt whether their catalyses still follow the established mechanism. As most of the early mechanistic studies of the *Pasturella* enzyme were performed on partially purified proteins, and the catalytic properties of the *Saccharopolyspora* enzyme had not been characterized, a re-examination of these enzymes at the homogeneous stage appeared to be in order. In an effort to study the mechanism of 3,6-dideoxyhexose formation in *Yersinia pseudotuberculosis* CDP-D-glucose oxidoreductase from this bacterial strain whose lipopolysaccharide structure is known to contain ascarylose (3,6-dideoxy-L-arabino-hexose) as the end group of its terminal subunit (22). However, examination of SDS-polyacrylamide gels of the proteins isolated by these well documented protocols clearly showed that our initial efforts were futile. This prompted us to develop a new separation procedure initiated by a DEAE-cellulose chromatography. Although most of the key enzymes involved in the biosynthesis of ascarylose were collected during the gradient elution (36, 39), the desired oxidoreductase was eluted only after washing isocratically with 200 mM of the potassium phosphate buffer. As summarized in Table I, further separation by Matrex Blue-A, hydroxylapatite, DEAE-Sephadex, Sephadex G-100, and NAD\(^+\)-agarose chromatography permitted a nearly 6000-fold overall purification of CDP-D-glucose oxidoreductase to homogeneity. This highly purified enzyme exhibited a single band upon SDS-polyacrylamide gel electrophoresis (Fig. 1).

**MATERIALS AND METHODS\(^3\)**

**RESULTS**

**Enzyme Purification**

Our initial attempts to purify CDP-D-glucose oxidoreductase from *Y. pseudotuberculosis* followed the procedures reported in the purification of analogous enzymes from other microbial origins, especially the *P. pseudotuberculosis* enzyme (22). However, examination of SDS-polyacrylamide gels of the proteins isolated by these well documented protocols clearly showed that our initial efforts were futile. This prompted us to develop a new separation procedure initiated by a DEAE-cellulose chromatography. Although most of the key enzymes involved in the biosynthesis of ascarylose were collected during the gradient elution (36, 39), the desired oxidoreductase was eluted only after washing isocratically with 200 mM of the potassium phosphate buffer. As summarized in Table I, further separation by Matrex Blue-A, hydroxylapatite, DEAE-Sephadex, Sephadex G-100, and NAD\(^+\)-agarose chromatography permitted a nearly 6000-fold overall purification of CDP-D-glucose oxidoreductase to homogeneity. This highly purified enzyme exhibited a single band upon SDS-polyacrylamide gel electrophoresis (Fig. 1).

\(^3\) *Pasturella pseudotuberculosis* is now reclassified as *Yersinia pseudotuberculosis* (18).

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**TABLE I**

| Purification step\(^a\) | Total protein\(^b\) | Total activity\(^c\) | Specific activity\(^d\) | Purification Yield \(^{-}\)fold | \(\%\) |
|------------------------|------------------|--------------------|----------------------|-------------------------------|------|
| Crude extracts\(^e\)   | 15,600           | 496                | 0.032                | 1                              | 100  |
| Streptomycin sulfate    | ND\(^f\)         | ND                 | ND                   | ND                            | ND   |
| Ammonium sulfate        | 6,680            | 438                | 0.066                | 2                              | 88   |
| DEAE-cellulose          | 190              | 224                | 1.18                 | 37                             | 45   |
| Matrex Blue-A           | 57               | 101                | 1.75                 | 55                             | 20   |
| Hydroxylapatite         | 5                | 88                 | 17.5                 | 550                            | 18   |
| DEAE-Sephadex           | 1.2              | 69                 | 57.5                 | 1797                           | 14   |
| Sephadex G-100          | 0.5              | 53                 | 106                  | 3313                           | 11   |
| NAD\(^+\)-agarose       | 0.25             | 47                 | 188                  | 5875                           | 9.5  |

\(^a\) Activity was assayed as detailed under “Materials and Methods.”

\(^b\) Protein in milligrams.

\(^c\) Micromoles of product formation per min.

\(^d\) Units per mg of protein.

\(^e\) Crude extracts were obtained from 95 g of wet cells.

\(^f\) ND, not determined.

**Fig. 1.** SDS-polyacrylamide gel electrophoresis of the purification of CDP-D-glucose oxidoreductase. Fractions of *Y. pseudotuberculosis* CDP-D-glucose oxidoreductase from various purification steps were analyzed by electrophoresis on a 13% SDS gel, and proteins were visualized by Coomassie Blue. From left to right: lane 1, Sephadex G-100 eluate; lane 2, NAD\(^+\)-agarose eluate (fractions 4–6); lane 3, NAD\(^+\)-agarose eluate (fractions 7–12); lane 4, M, markers (bovine serum albumin (66,000), ovalbumin (45,000), carbonic anhydrase (29,000), and α-lactalbumin (14, 200)); lane 5, DEAE-Sephadex eluate; lane 6, hydroxylapatite eluate; lane 7, Matrex Blue-A eluate; lane 8, eluate from DEAE-cellulose chromatography. This analysis was performed as described under “Materials and Methods.”

**Properties of CDP-D-glucose Oxidoreductase**

**Molecular Weight**—The native molecular weight of the highly purified CDP-D-glucose oxidoreductase was estimated by gel filtration to be 86,000, while SDS-polyacrylamide gel electrophoresis of this pure protein showed a single band with a molecular weight of 42,500. It is apparent that this oxidoreductase is a dimeric protein consisting of two identical subunits.

**UV-visible Spectrum**—The electronic spectrum of CDP-D-glucose oxidoreductase is a spectrum of a single polypeptide which shows no absorption at wavelength above 300 nm.

**Amino Acid Composition and Amino-terminal Sequence**—The amino acid composition and the amino-terminal sequence are summarized in Table II. This sequence has been used in designing an oligonucleotide primer as a probe for cloning this enzyme’s gene.

**Substrate Specificity**—The specificity of CDP-D-glucose oxidoreductase for CDP-D-glucose was examined by substituting alternate substrates for CDP-D-glucose in the normal assay. In this study, ADP-D-glucose, GDP-D-glucose, TDP-D-gluc-
CDP-D-glucose Oxidoreductase from Y. pseudotuberculosis

**TABLE II**

| Residue | Residues/molecule | Residue | Residue/molecule |
|---------|------------------|---------|-----------------|
| Asx     | 36.9             | Ile     | 17.9            |
| Thr     | 20.1             | Leu     | 29.0            |
| Ser     | 16.2             | Tyr     | 8.6             |
| Glx     | 36.7             | Phe     | 7.9             |
| Pro     | 12.0             | Lys     | 18.6            |
| Gly     | 31.6             | His     | 10.9            |
| Ala     | 31.6             | Arg     | 16.0            |
| Met     | 10.6             | Cys     | 4.5             |
| Val     | 26.8             |         |                 |

**Fig. 2.** Effect of NAD⁺ concentration upon CDP-D-glucose oxidoreductase activity. Illustrated is the dependence of CDP-D-glucose activity (O) in response to increasing amounts of NAD⁺. This experiment was performed as described under “Materials and Methods.”

The amino-terminal amino acid sequence was Met-Ile-Asn-Asn-Ser-Phe-Trp-Gln-Gly-Lys-Arg-Phe-Phe-Val-Thr-Val.

The deuterium content estimated by NMR by reference to the unlabeled sample of the reduced product 7 (43) was greater than 95%. Inversion at C-3 of 7 to afford the d-glucose configuration was effected by displacing the C-3 tosyl group with sodium benzoate in hot N,N-dimethylformamide (44). As the benzoate substituent of 8 was found to be labile in the subsequent reactions, it was replaced with the more stable benzyl protecting group. Stereospecific tritium incorporation at C-6 was the most challenging step since the C-5 hydroxymethyl group is not rigidly held within the sugar’s ring structure. The method eventually chosen was the one established by Kakinuma (45, 46), which is in many ways analogous to the classical synthesis of chiral acetates developed by Cornforth et al. (47). The common precursor, a 5,6-yne derivative 13, was obtained from the dibromo olefin 12 upon treatment with n-butyllithium in THF at -78 °C followed by quenching with [3H₂]H₂O (30 mCi, 0.3 ml) (48). Transformation of 13 with a specific radioactivity of 0.46 Ci/mol to the (E)-olefin 14 was achieved by reduction using chromous sulfate in aqueous N,N-dimethylformamide (49). 1H NMR analysis of a control using the 6-'H₁-labeled 13 as the reactant revealed that this reaction proceeded with 91% stereospecificity. Di-hydroxylation with a catalytic amount of OsO₄ in the presence of N-methylmorpholine N-oxide (NMO) afforded the desired cis diol 15 in 72% yield (50). A small albeit noticeable amount of the (5S,6R)-isomer was also formed, but was readily removed by flash chromatography. The protected glucose was diluted 5-fold with 9 and was converted to free (6S)-D-[4-3H₂,6-3H]glucose (3a) by hydrogenolysis (10% Pd/C) and subsequent acid hydrolysis. The 6R-labeled glucose was prepared analogously from 13 via the (Z)-olefin 16 which was produced by hydrogenation over Lindlar catalyst in the presence of sodium benzoate in hot N,N-dimethylformamide (44). 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Transformation of 13 with a specific radioactivity of 0.46 Ci/mol to the (E)-olefin 14 was achieved by reduction using chromous sulfate in aqueous N,N-dimethylformamide (49). 1H NMR analysis of a control using the 6-'H₁-labeled 13 as the reactant revealed that this reaction proceeded with 91% stereospecificity. Di-hydroxylation with a catalytic amount of OsO₄ in the presence of N-methylmorpholine N-oxide (NMO) afforded the desired cis diol 15 in 72% yield (50). A small albeit noticeable amount of the (5S,6R)-isomer was also formed, but was readily removed by flash chromatography. The protected glucose was diluted 5-fold with 9 and was converted to free (6S)-D-[4-3H₂,6-3H]glucose (3a) by hydrogenolysis (10% Pd/C) and subsequent acid hydrolysis. The 6R-labeled glucose was prepared analogously from 13 via the (Z)-olefin 16 which was produced by hydrogenation over Lindlar catalyst in the presence of sodium benzoate in hot N,N-dimethylformamide (44).
of quinoline. Conversion of the 6S- and 6R-labeled glucose to the corresponding CDP derivatives was accomplished by a sequence commonly used to make nucleoside diphosphohexoses (28, 29). The CDP-D-glucose pyrophosphorylase used in this incubation was partially purified from the same strain of \textit{Y. pseudotuberculosis}. The identity of the product was confirmed by comparing its HPLC retention time with that of authentic CDP-D-glucose. The specific radioactivities of the final 6S and 6R products were 89 and 74 mCi/mol, respectively, and the overall yields were 18-20%.

**Chiral Methyl Analysis of Acetate Samples Derived from Product of Enzyme Incubation—**The (6S)- and (6R)-CDP-D-[4-3H,6-3H]glucose were each mixed with CDP-D-[U-14C]glucose, diluted with excess unlabeled CDP-D-glucose (1:9), and then incubated with homogeneous CDP-D-glucose oxidoreductase in the presence of \textit{NAD}-. The CDP-4-keto-6-deoxy-D-glucose products isolated by paper chromatography (EtOH/H$_2$O = 5:5:2) were subjected to Kuhn-Roth oxidation (51, 52). The nascent acetic acid samples were formed in radiochemical yields of 52-54%, and their chiralities were determined by the method of chiral methyl analysis (47, 53-55). An \textit{F} value (56) of 71 corresponding to a 72% ee \textit{R} configuration and an \textit{F} value of 30 corresponding to a 69% ee \textit{S} configuration were obtained for the two acetates derived from the 6S- and 6R-labeled glucose, respectively. The same analysis was also performed on chiral \textit{S} and \textit{R} configuration acetate standards which were prepared from tritium labeled glycine. The numbers obtained for all acetates analyzed are summarized in Table III.

**DISCUSSION**

The conversion of nucleotide diphosphohexose to its 4-keto-6-deoxyhexose derivative has been shown to be the first step unique to the biosynthesis of several naturally occurring deoxy sugars. In an attempt to investigate the formation of ascorbylone, a bacterial antigentic determinant, we have purified a CDP-D-glucose oxidoreductase catalyzing the first bio-synthetic step of this 3,6-dideoxyhexose from \textit{Y. pseudotuberculosis}. Although evidence for the occurrence of isozymes had been reported for TDP-D-glucose oxidoreductase found in \textit{Salmonella typhimurium}, gel electrophoresis of our enzyme revealed only a single enzymatically active band with identical mobility at all stages of purification. This purified enzyme alone was able to catalyze the conversion of CDP-D-glucose to CDP-4-keto-6-deoxy-D-glucose, and incubation of the protein with \textit{NAD}+ followed by gel filtration failed to reconstitute the enzyme activity in full. Such an absolute requirement of \textit{NAD}+ for its activity, as illustrated in Fig. 2, makes this enzyme a rare example among its class of oxidoreductases (12).

Since studies of the catalysis of two other sugar oxidoreductases, TDP-D-glucose oxidoreductase (29) and GDP-D-mannose oxidoreductase (31), have shown a remarkable stereochemical convergency in which the displacement of C-6 hydroxyl group by C-4 hydrogen proceeds intramolecularly with inversion of configuration, an analogous analysis directed at elucidating the stereochemical course of the reaction mediated by CDP-D-glucose oxidoreductase may provide unique mechanistic insights that are not available from other experimental approaches. In order to carry out the proposed stereochemical study, a set of stereospecifically labeled sugar nucleotide substrates and acetate standards were synthesized, mainly by chemical reactions. Although these compounds had been prepared heretofore by methods based on complex enzymatic manipulations (29), the large quantities of material and the well defined chiral purity of the sample provided by a chemical synthesis made this new approach uniquely appealing. As shown in Scheme II, the precursors of the substrate, 3a and 3b, were prepared so that every tritiated molecule also carried a deuterium at C-4. If the displacement of the C-6 hydroxyl group by the migrating C-4 deuterium is indeed stereospecific, the resulting methyl group at C-6 is expected to contain hydrogen, deuterium, and tritium in a chiral arrangement of either \textit{S} or \textit{R} configuration. Kuhn-Roth oxidation of the incubation product 2 will give an acetic acid derived from the \textit{C} and \textit{C} of the sugar moiety, and the chirality of the methyl group can then be determined by the method of Cornforth et al. (53) and Lüthy et al. (54). The key step of this well established analysis is the malate synthase-mediated coupling of the acetate in its coenzyme A form with glyoxylate to form malate. Due to an isotope effect of 3.7-3.8 exhibited by this condensation, the malate derived from \textit{R} and \textit{S} labeled acetate will have a level of tritium labeling at C-5 dependent on the chirality of the original acetate. The unsymmetrical tritium distribution between the two diastereotopic hydrogens at C-3 of malate can be determined by incubating with fumarase, which catalyzes the stereospecific exchange of the pro-3R hydrogen with solvent protons. Using this method, chirally pure acetates of \textit{R} and \textit{S} configuration are expected to give \textit{F} values (percentage of tritium retention in the fumarase reaction) of 79 and 21, respectively (58).

As shown in Table III, the malate derived from 6R-labeled glucose (3b) retained only 30% of its tritium after equilibration with fumarase, while the malate derived from 6S-glucose (3a) retained 71% of its tritium. These \textit{F} values clearly indicate that incubation of the 6S isomer (3a) with the purified oxidoreductase gave a product containing a stereospecifically labeled methyl group of \textit{R} configuration, whereas the 6R isomer (3b) yielded material bearing a chiral methyl group of \textit{S} configuration. In light of the large dilution of the labeled precursor by the unlabeled species prior to the incubation with oxidoreductase, the aforementioned results confirm that this enzymatic conversion involves an intramolecular hydrogen migration from C-4 to C-6, since the methyl

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*H. Liu and T. M. Weigel, unpublished results.*

**TABLE III**

| Compound analyzed | (6S)-CDP-D-[4-3H,6-3H]glucose | (6R)-CDP-D-[4-3H,6-3H]glucose |
|-------------------|-------------------------------|-------------------------------|
|                   | \(^{3}H\) | \(^{14}C\) | \(^{3}H^{14}C\) ratio | \(^{3}H\) | \(^{14}C\) | \(^{3}H^{14}C\) ratio |
| Acetate (from Kuhn-Roth oxidation) | 10,580 | 5,929 | 1.79 | 33,880 | 20,085 | 1.69 |
| Malate | 2,068 | 1,539 | 1.34 | 6,741 | 5,464 | 1.23 |
| Malate/fumarate (after equilibration) | 1,463 | 1,529 | 0.957 | 2,023 | 5,434 | 0.372 |
| Tritium retention in the fumarase reaction | 71.4% | 30.2% |
| Configuration of the acetate | \(R\) | \(S\) |
The initially formed anion is commonly and in many enzymatic reactions where the elimination is the displacement of the C-6 hydroxyl group by the hydrogen exhibits a mechanism analogous to that proposed for other deuterium from C-4 to C-6 is strictly intramolecular and mainly by the lifetime of the initially formed carbanion since the mechanism of an elimination reaction is determined by the stereochemical mode of the dehydration of configuration at C-6, the removal of water from C-5 and C-6 is not fully stereospecific. Obviously, more experiments are needed to gain further insights into this mechanistic ambiguity. It is worth mentioning that the chiral methyl group at C-6 generated by GDP-d-mannose oxidoreductase showed an even lower degree of chiral purity (31). Thus, this enzyme may have an even weaker binding for its coenzyme. However, since the later analysis was performed with a cell-free extra-enzyme oxidation reaction, no information is available. Namely, occasional cofactor dissociation may have led to lower chiral purity of the product due to the replacement of deuterated cofactor midway through the catalytic cycle with unlabeled NADH from the cellular pool.

In conclusion, an NAD\(^{+}\)-linked oxidoreductase catalyzing the conversion of CDP-d-glucose to the corresponding 4-keto-6-deoxy-d-glucose derivative was isolated from Y. pseudotuberculosis. It belongs to a small group of enzymes that are NAD\(^{+}\)-dependent even though the overall catalysis is redox in nature (12, 60). Unlike most other enzymes in this class that have a tightly bound NAD\(^{+}\) in the active site, the purified enzyme showed an absolute requirement for exogenous NAD\(^{+}\) following purification. Despite its low cofactor affinity, the stereocchemical analysis presented herein using chemically synthesized labeled substrate of high enantiomeric purity clearly showed that this purified enzyme undergoes a mechanism consistent with that followed by other members of its class. Aside from the moderate enantioselection found for the acetate samples, the mechanism and stereocchemical convergency exhibited by CDP-D-glucose oxidoreductase with all of the other sugar oxidoreductases characterized so far suggested that this class of enzymes, regardless of their source, may evolve from a common progenitor whose catalytic course has persevered throughout the enzyme's subsequent diversification.

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Step 3. Western Blot Analysis of C. glutamicum Chromosomes. The antibody selected for step 3 (100 ml) was applied to a Western blot analysis with 30 and 50 hits, respectively. The results are shown in Figure 3B. The Western blot analysis of the 30 hits and 50 hits was conducted using the same electrophoretic conditions. The results are shown in Figure 3B. The Western blot analysis of the 30 hits and 50 hits was conducted using the same electrophoretic conditions.

Antibody and Conjugate and Ampere-Terminal Analysis. The antibody and conjugate were determined by a Western blot analysis with the Western blot analysis with 30 and 50 hits, respectively. The results are shown in Figure 3B. The Western blot analysis of the 30 hits and 50 hits was conducted using the same electrophoretic conditions.

Step 4. DEAE-cellulose Column Chromatography. The enzyme solution from step 3 (100 ml) was applied to a DEAE-cellulose column with 30 and 50 hits, respectively. The results are shown in Figure 3B. The Western blot analysis of the 30 hits and 50 hits was conducted using the same electrophoretic conditions.
CDP-D-glucose Oxidoreductase from Y. pseudotuberculosis

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60. 1-(a-hemiacetal)-3-O-methyl-D-glucose (80 mg, 0.17 mmol) and p-nitrobenzoic acid (40 mg, 0.22 mmol) were added to a suspension of NaOH (1 M, 1.4 mL) with stirring. The solution was evaporated to dryness, giving 128 mmol in 98% yield. At this point, the compound had a specific activity of 8.8 T.AU/mmol.

Conversion of labelled CDP-D-glucose to the corresponding CDP-4-keto-6-phosphogluconate was carried out by heating 1.3 mg of the reduced CDP-D-glucose to a final concentration of 10 mg/mL in 0.1 M phosphate buffer pH 7.0 at 37 °C. The reaction mixture was heated for 1 h and then cooled on ice. The reaction mixture was then chromatographed on a Whatman P50-200 paper using a solvent system of ethyl acetate:acetone:water (50:50:15) to remove the unreacted starting material. The chromatographed reaction mixture was then eluted with the same solvent system to remove the unreacted starting material. The chromatographed reaction mixture was then eluted with the same solvent system to remove the unreacted starting material. The chromatographed reaction mixture was then eluted with the same solvent system to remove the unreacted starting material. The chromatographed reaction mixture was then eluted with the same solvent system to remove the unreacted starting material.