D-Fucose Metabolism in a Pseudomonad

III. CONVERSION OF D-FUCONATE TO 2-KETO-3-DEOXY-D-FUCONATE BY A DEHYDRATASE*

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SUMMARY

An aldonic acid dehydratase was shown to be instrumental in the metabolism of D-fucose in a pseudomonad. The enzyme was purified 30-fold, and some of its properties are reported. Of 19 aldonic acids tested, only D-fuconate and L-arabonate served as substrates. The Km values for D-fuconate and L-arabonate were 4.0 mM and 4.3 mM, respectively. The product of D-fuconate dehydration was identified as 2-keto-3-deoxy-D-fuconate. A D-fuconate dehydratase-less mutant was isolated and shown to grow normally on D-glucose and D-galactose, but not to grow on D-fucose or L-arabinose.

The preceding papers in this series described the enzymatic oxidation of D-fucose to D-fuconate by enzymes from a pseudomonad selected for its ability to use D-fucose as a sole carbon and energy source (1, 2). The present paper documents that the next step in D-fucose metabolism is the dehydration of D-fuconate to yield 2-keto-3-deoxy-D-fuconate (Fig. 1), and reports some properties of the dehydratase that catalyzes the reaction. The following paper (3) describes the further metabolism of 2-keto-3-deoxy-D-fuconate.

MATERIALS AND METHODS

Dehydratase Assay—The standard assay was a modification of the semicarbazone method of MacGee and Doudoroff (4). The reaction mixture (0.15 ml) contained 20 μmoles of Bicine buffer (pH 7.2), 0.15 μmole of 2-thioethanol, 5.0 μmoles of D-fuconate (or other substrate where noted under “Results”), 5.0 μmoles of MgCl₂, and enzyme. The mixture was incubated at 30° for 30 min, and then was quenched by the addition of 1.0 ml of the semiacarbamide reagent. After a 15 min incubation at 30°, the mixture was diluted to 5.0 ml with water and the absorbance was read at 250 nm in a 1.0-cm quartz cuvette against a diluted reagent blank. Controls were necessary to correct for the absorbance of protein and other reaction components. The rate of product formation was constant with time and proportional to enzyme concentration. A unit of dehydratase was defined as the amount that catalyzed the formation of 1 μmole of α-keto acid per min, calculated on the basis of a molar absorption coefficient of 10,200 (4).

In some cases, where a rapid qualitative assay for 2-keto-3-deoxyaldonic acid formation was desired (as in scanning fractions from chromatographic columns), the periodate-thiobarbituric acid procedure of Weissbach and Hurwitz (5) was used.

Analytical Methods—2-Keto-3-deoxyaldonic acids were measured with the 2-thiobarbiturate-periodate procedure (5). Acetalddehyde was determined by the method of Barker and Summers (6), using periodate removal techniques of Itagaki and Suzuki (7). CO₂ from ceric sulfate decarboxylation of carboxylic acids was measured manometrically (8). Carbonyl compounds were determined as the tetrazopentamethine cyanine derivatives (9). Sodium borohydride reduction was performed by the method of Ghalambor et al. (10). Periodate consumption was determined by the method of Dixon and Lipkin (11) as modified by Sugimoto and Okazaki (12). Absorption spectra were recorded with a Cary model 15 spectrophotometer. Other analytical procedures were those used in Paper I (1).

Reagents—2-Keto-3-deoxy-n-galactonate and 2-keto-3-deoxy-D-glucuronate were prepared by alkaline phosphatase treatment of their respective 6-phospho esters, which were gifts from Dr. W. A. Wood. D-Lactaldehyde and D,L-lactaldehyde were prepared from L-threonine and D,L-threonine, respectively, by the method of Zagalak et al. (13). Potassium salts of aldonic acids were prepared from their corresponding aldoses by the method of Moore and Link (14). D-Glucarate and D-galactarate, obtained from Sigma, were crystallized as the dicyclohexylammonium salts (15) prior to use. 2-Thiobarbituric acid, obtained from Eastman, was also recrystallized (16). Other chemicals were obtained from standard commercial sources or as described previously (1).

RESULTS

Purification of D-Fuconate Dehydratase

Extracts (1) were prepared from L-arabinose-grown cells of pseudomonad MSU-1 suspended in 0.10 M Bicine buffer (pH 7.2) or N,N-bis(2-hydroxyethyl) glycinene; HEPES, N,N-bis(2-hydroxyethyl)piperazine-N'-2-ethanesulfonate.
Fig. 1. Reaction for the dehydration of D-fuconate to 2-keto-3-deoxy-D-fuconate by D-fuconate dehydratase.

**Table I**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (ml)</th>
<th>Total Protein (mg)</th>
<th>Total Activity (units)</th>
<th>Specific Activity (units/mg)</th>
<th>A260</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell extract</td>
<td>380</td>
<td>8435</td>
<td>650</td>
<td>0.0770</td>
<td>0.68</td>
</tr>
<tr>
<td>Protamine sulfate supernatant</td>
<td>455</td>
<td>6598</td>
<td>648</td>
<td>0.0983</td>
<td>0.84</td>
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<tr>
<td>(NH4)2SO4 precipitate</td>
<td>75</td>
<td>1573</td>
<td>491</td>
<td>0.312</td>
<td>1.22</td>
</tr>
<tr>
<td>Sephadex G-200</td>
<td>79</td>
<td>869</td>
<td>117</td>
<td>1.35</td>
<td>1.48</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>20</td>
<td>16</td>
<td>37.3</td>
<td>2.33</td>
<td>1.60</td>
</tr>
</tbody>
</table>

Properties of D-Fuconate Dehydratase

The DEAE-cellulose fraction was used in the following experiments, unless specified otherwise.

**pH Optimum**—D-Fuconate dehydratase activity as a function of pH was maximal at pH 7.1 to 7.4 in Bicine and HEPES buffers (Fig. 2). When 2-thioethanol was omitted from the reaction mixture, activity was decreased at pH values below 7.7, but was increased at pH values above pH 7.7 (Fig. 2).

**Substrate Specificity and Kinetic Constants**—Of 10 aldonic acids tested at a concentration of 60 mM, only D-fuconate and L-arabonate served as substrates for the dehydratase. Compounds that did not serve as substrates (<2% of the rate with D-fuconate) were: L-fuconate, D-arabonate, D-galactonate, D-ido-6-deoxy-D-galactonate, D-ribonate, D-xylonate, D-lyxonate, L-mannonate, L-ribofuranose, D-ribofuranose, D-galactonate, D-galactoarabinose, D-glucuronate, D-glucuronate, and cellobiose.

From Lineweaver-Burk plots, the $K_m$ values for D-fuconate and L-arabonate were found to be 4.0 mM and 4.3 mM, respectively. The relative $V_{max}$ values were 0.47 and 1.00, respectively.

**Effect of Metal Ions on Dehydratase Activity**—Dehydratase activity was nil in the presence of 0.33 mM EDTA. The further addition of various salts (13.3 mM) resulted in the following relative rates (per cent of original activity): MgCl$_2$ or MnSO$_4$, 100; MnCl$_2$, 85; FeSO$_4$, 37; CuSO$_4$, 28; CoCl$_2$, 18; NiCl$_2$, 8; CaCl$_2$, 0; ZnCl$_2$, 0. The optimal Mg$^{2+}$ concentration was about 20 mM (Fig. 3).

**Effect of Thiols and Thiol Group Reagents**—When any of various thiols (2-thioethanol, reduced glutathione, or dithiothreitol) were added to the dehydratase assay mixture at 2.7 to 2.8 mM, the rate of the reaction was 30 to 45% greater than in their absence. In the absence of added thiol compounds, 2.2 mM sodium iodoacetate had no significant effect on the reaction velocity, but 0.05 mM p-chloromercuribenzoate caused 88% inhibition.
Stability—The purified enzyme was stable unfrozen at 0–4° (<10% loss per week), but was about 30% inactivated when stored frozen overnight. It was also readily inactivated at 53° (half-life of about 80 sec when assayed with either n-fuconate or L-arabinose as the substrate).

Identification of Product of n-Fuconate Dehydratase-catalyzed Reaction

Enzymatic Synthesis of 2-Keto-3-deoxy-n-fuconate—The reaction mixture (10.0 ml) contained 500 μmoles of potassium n-fuconate, 50 μmoles of MgCl₂, and n-fuconate dehydratase (20 mg of protein, specific activity, 13.5). The pH was adjusted to 7.4. After several hours of incubation at 25°, the reaction mixture was passed through a column (0.5 x 35 cm) of Dowex 50W-H⁺, and the nonionic material was collected by washing the column with 750 ml of deionized water. The product in the eluate was further purified by chromatography on a column (0.5 x 35 cm) of Dowex 1-X8 formate (200 to 400 mesh), using a linear gradient of 0 to 0.08 M formic acid (1000 ml). A single 2-thiobarbituric-acid-positive peak was obtained. The fractions were pooled and concentrated to a syrup under reduced pressure at 30° to remove the formic acid. The syrup (the n-fuconate dehydration product) was dissolved in water, and converted to the potassium salt with Dowex 50W-K⁺. This solution was then lyophilized to form an amorphous solid. The yield of potassium 2-keto-3-deoxy-n-fuconate was 490 μmoles (98%).

Chemical Synthesis of 2-Keto-3-deoxy-n-fuconate and 2-Keto-3-deoxy α-fuconate—These compounds were synthesized by modification of the method used for the synthesis of N-acetylsuccinimic acid (18). The reaction involves an aldol condensation of lactaldehyde with a pyruvyl carbanion generated by alkaline dehydrogenase.

D- or L-lactaldehyde (0.34 g) was dissolved in 20 ml of water, and the pH was adjusted to 7.2. Oxalacetate (0.226 g) was dissolved gradually in 2.0 ml of water by maintaining the pH at 8.5 with a Sargent pH stat. The lactaldehyde solution was then added to the oxalacetate solution, and the pH was adjusted to and maintained at 11.0 at 25°. The reaction was monitored by measuring 2-keto-3-deoxy acid formation with the 2-thiobarbituric acid procedure. After 20 min, when the reaction was complete, the product was converted to the acid form by treatment with Dowex 50W-H⁺. The solution was desalted by the method of Hershberger et al. (20), and then applied to a column (0.5 x 15 cm) of Dowex 1-X8 formate by the method of Samuelsen et al. (21).

The compounds co-eluted as one peak in each of the following: chromatography on a column of Dowex 1-X8 formate by the procedure described above; chromatography on a column of Dowex 1-X8 carbonate by the method of Hershberger et al. (20); and chromatography on Dowex 1-X8 borate by the method of Samuelson et al. (21).

The compounds co-chromatographed as one periodate-thiobarbituric acid-positive (22) spot on Whatman No. 1 paper in the following solvents: (a) 1-propanol-formic acid-water (6:3:1), and (b) 1-butanol-pyridine-water (6:4:3). The R₂ values were 0.29 and 0.78, respectively.

The dehydrogenase product and synthetic 2-keto-3-deoxyfuconate were each derivatized with compounds that react with α-keto acids to form characteristic chromogens. Reaction with 2-thiobarbituric acid-positive (22) spot on Whatman No. 1 paper in the following solvents: (a) 1-propanol-formic acid-water (6:3:1), and (b) 1-butanol-pyridine-water (6:4:3). The R₂ values were 0.29 and 0.78, respectively.

The dehydorogenase product and synthetic 2-keto-3-deoxyfuconate were each derivatized with compounds that react with α-keto acids to form characteristic chromogens. Reaction with 2-thiobarbituric acid-positive (22) spot on Whatman No. 1 paper in the following solvents: (a) 1-propanol-formic acid-water (6:3:1), and (b) 1-butanol-pyridine-water (6:4:3). The R₂ values were 0.29 and 0.78, respectively.

Treatment of the compounds with periodate and 2-thiobarbituric acid (5) gave a colored compound with a λₘₐₓ of 551 nm, and which was unstable in alkaline. This is characteristic of a 2-thiobarbituric acid-β-formylpyruvate complex (10, 16); the β-formylpyruvate is a characteristic product of the reaction of periodate with 2-keto-3-deoxy-aldoic acids (5). The amount of periodate consumed was 0.89 mole per mole of dehydorogenase product or chemically prepared 2-keto-3-deoxyfuconate.

The compounds were reduced with NaBH₄ and the reduction products were then oxidatively decarboxylated with ceric sulfate. One mole of CO₂ was formed per mole of reactant. The reduction products were 2-thiobarbituric acid-negative; this was expected, since β-formylpyruvate resulting from periodate oxidation of the mixed metasaccharinic acids (reduction products of 2-keto-3-deoxy-fuconate) does not react with 2-thiobarbituric acid and shown to serve as a substrate for NADH-linked lactate dehydrogenase.
acid to form a chromogen (25). However, after ceric sulfate oxidation, a compound was formed which was 2-thiobarbituric acid-positive, giving a \( \lambda_{\text{max}} \) at 532 nm. The chromogen was consistent with the dehydration product having a keto group at C-2 and a methylene group at C-3.

Evidence that the configuration of the hydroxyl groups of the dehydratation product are threo rather than erythro was obtained by comparing the rate of periodate oxidation with the rate observed for authentic threo and erythro 3-deoxyhexuloseic acids. The periodate oxidation rates for the dehydration product, chemically prepared 2-keto-3-deoxy-D-fuconate, and 2-keto-3-deoxy-D-galactonate (3-deoxy-D-threo-hexulosonate) were identical, and were slower than the rate for 2-keto-3-deoxy-L-gluconate (3-deoxy-L-erythro-hexulosonate) (Fig. 4). Thus, it is concluded that the configuration of the hydroxyl groups at C-4 and C-5 of D-fuconate remained unchanged during dehydration at C-2 and C-3.

Finally, the rate of cleavage of the dehydration product by 2-keto-3-deoxy-D-fuconate aldolase (3) was the same as that of chemically synthesized 2-keto-3-deoxy-D-fuconate. In an end point assay, the products of the aldolase-catalyzed cleavage of the dehydratation product and chemically synthesized 2-keto-3-deoxy-D-fuconate were 0.98 mole of pyruvate and 0.96 mole of lactaldehyde per mole of substrate, whereas aldolase-catalyzed cleavage of 2-keto-3-deoxy-DL-fuconate yielded only 0.48 mole of pyruvate per mole of product. Thus, the D-fuconate dehydratase reaction product is established as 2-keto-3-deoxy-D-fuconate.

**Inducibility of D-Fuconate Dehydratase**

Cells were harvested after 18 hours of growth on various substrates. Cell extracts were prepared in 0.01 M Bisce buffer (pH 7.4) and 0.14 mm 2-thioethanol. Specific activities of D-fuconate dehydratase in crude extracts were (growth substrate in parentheses): 0.78 (D-fucose), 0.71 (L-arabinose), 0.71 (D-galactose), 0.09 (D-glucose), and 0.06 (nutrient broth). Thus, the enzyme activity was increased about 12-fold by growth on precursors of its substrates (D-fucose and L-arabinose) over the level in nutrient broth-grown cells.

**Isolation and Characteristics of a D-Fuconate Dehydratase Mutant**

The wild type culture (MSU-1) was mutagenized with ethyl methanesulfonate by minor modifications of standard procedures (26) and was replicated on Petri plates containing D-glucose- and L-arabinose-mineral agar. A mutant which was L-arabinose-negative and D-glucose-positive was isolated. A revertant was then selected from the mutant by plating on L-arabinose-mineral agar.

The mutant grew normally on D-glucose and D-galactose (doubling time of about 3 hours at 32°) but, unlike the wild type (1), failed to grow on L-arabinose or D-fucose. The revertant grew normally on all four sugars.

Table II shows activities of relevant enzymes in cell extracts of all three strains. It can be seen that D-fuconate dehydratase activity is deficient in the mutant and has been regained in the revertant. The other three enzymes involved in D-fuconate metabolism—D-fuconate dehydrogenase (1), L-arabinose-aldo dehydrogenase (2), and 2-keto-3-deoxy-L-arabinionate aldolase (3)—are essentially unchanged in all three strains. D-Galactonate dehydratase activity also showed no change.

**Discussion**

The quantitative conversion of D-fucose to a substance which, after periodate oxidation, yielded a compound that reacted with 2-thiobarbituric acid to form a chromogen with a \( \lambda_{\text{max}} \) of 551 nm indicated that the enzyme active on D-fucose was a dehydratase. The reaction product was further identified as 2-keto-3-deoxy-D-fuconate by its reactions with semicarbazide, o-phenylenediamine, and 3-methyl-2-hexothiazolinone hydrazide; by its reaction with periodate (rate studies, amount of periodate consumed, and identification of the products); by reaction of its borohydride reduction product with ceric sulfate; by analysis of the products after aldolase-catalyzed cleavage; and by comparison of its chromatographic behavior with that of chemically synthesized 2-keto-3-deoxy-D-fuconate. Since D-fucose is oxidized to D-fuconate in this organism (1, 2), it is thus established that 2-keto-3-deoxy-D-fuconate is an intermediate in D-fucose metabolism. The demonstration that a mutant missing the dehydratase grows normally on D-glucose and D-galac-
tose but fails to grow on D-fucose further confirms the participation of this enzyme in D-fucose metabolism. The fact that the dehydrataseless mutant also fails to grow on L-arabinose indicates that this same enzyme is the dehydratase previously proposed to function in L-arabinose metabolism in this organism (27). The thermal denaturation data are also consistent with this view.

D-Fuconate and L-arabonate are the only compounds tested that served as substrates. Considering structural similarities, it is somewhat surprising that the enzyme did not catalyze the dehydration of D-galactonate. D-Galactonate dehydratase activity has been found in extracts of D-galactose- and D-fucose-grown cells, but clearly it is not the same enzyme that acts on D-fuconate. L-Arabonate dehydratase activity has been detected in crude extracts of other pseudomonads (28, 29), but the enzymes from those sources have not been purified, and it is not known if they act on D-fuconate.

Since D-galactose (or D-galactonate?) appears to induce D-fucose dehydratase in this organism, but D-galactonate does not serve as a substrate for the enzyme, D-galactose may be considered to be a gratuitous inducer. This is opposite the situation that exists in Escherichia coli, for which D-fucose is a gratuitous inducer of the D-galactose operon (30).

The next reaction in D-fucose metabolism, the aldol cleavage of 2-keto-3-deoxy-D-fuconate to yield pyruvate and D-lactaldehyde, is the subject of the following paper (3).

REFERENCES

d-Fucose Metabolism in a Pseudomonad: III. CONVERSION OF d-FUCONATE TO 2-KETO-3-DEOXY-d-FUCONATE BY A DEHYDRATASE
A. Stephen Dahms and Richard L. Anderson


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