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8-Ketodeoxycoformycin and 8-Ketocoformycin as Intermediates in the Biosynthesis of 2′-Deoxycoformycin and Coformycin†

Jeffery C. Hanvey,‡,§ Elma Smal Hawkins,‖,⊥ David C. Baker,‖ and Robert J. Suhadolnik*,‡
Department of Biochemistry, Temple University School of Medicine, Philadelphia, Pennsylvania 19140, and the Department of Chemistry, University of Alabama, Tuscaloosa, Alabama 35487-9671

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ABSTRACT: An enzyme has been isolated from cell-free extracts of *Streptomyces antibioticus* that can catalyze the reduction of 8-ketodeoxycoformycin (8-ketodCF) and 8-ketocoformycin (8-ketoCoF) to the naturally occurring nucleoside analogues 2′-deoxycoformycin (dCF) and coformycin (CoF), respectively. The partially purified reductase requires NADPH as the cofactor and stereospecifically reduces the 8-keto group of both ketonucleoside substrates to a hydroxyl group with the *R* configuration at C-8. This is the same configuration of the hydroxyl group as that of the dCF and CoF isolated from *S. antibioticus*. The reduction proceeds at the nucleoside level, and ATP is not required. The reductase is stereospecific for the NADPH cofactor in that it transfers the *pro-S* but not the *pro-R* hydrogen from C-4 of NADPH to the 8-keto group. The apparent K_m for 8-ketodCF and 8-ketoCoF were 250 and 150 μM, respectively. These in vitro results, which show that 8-ketodCF and 8-ketoCoF may be intermediates in the biosynthesis of dCF and CoF, support and extend our earlier results from in vivo studies which established that adenosine and C-1 of D-ribose are the carbon–nitrogen precursors of dCF. A possible mechanism for the formation of dCF is presented.

2′-Deoxycoformycin (dCF)[1] and coformycin (CoF) are nucleoside antibiotics produced in trace amounts by *Streptomyces antibioticus* (Ryder et al., 1975; Woo et al., 1974). The heterocyclic moiety of these compounds, which contains a 1,3-diazepine ring, is similar to that of a purine ring except that an additional CH_2 is located between N-1 and C-6 (Figure 1A).

dCF has been used in the treatment of T-cell acute lymphoblastic leukemia [for review see *Cancer Treatment Symposia* (1984)], chronic lymphoblastic leukemia (Grever et al.,

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‡ Temple University School of Medicine.
§ Present address: Department of Biochemistry, University of Alabama at Birmingham, Birmingham, AL 35294.
‖ University of Alabama.
⊥ Present address: Parke-Davis Pharmaceutical Research, 2800 Plymouth Road, Ann Arbor, MI 48105.

[1] Abbreviations: EDTA, (ethylenedinitrilo)tetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PRPP, 5-phosphoribose 1-pyrophosphate; Tris, tris(hydroxymethyl)aminomethane. Other names for 2′-deoxycoformycin (dCF) include covidarabine (CoV) and pentostatin (USAN). The systematic name is (8*R*)-3-(2-deoxy-β-D-*erythro*-pentofuranosyl)-3,6,7,8-tetrahydroimidazo[4,5-*d*][1,3]diazepin-8-ol. The systematic name for coformycin (CoF) is (8*R*)-3-(β-D-ribofuranosyl)-3,6,7,8-tetrahydroimidazo[4,5-*d*][1,3]diazepin-8-ol.

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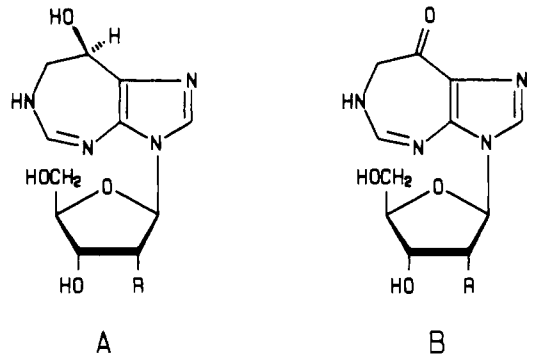

FIGURE 1: (A) R = OH, coformycin (CoF); R = H, 2'-deoxycoformycin (dCF). (B) R = OH, 8-ketocoformycin (8-ketoCoF); R = H, 8-ketodeoxycoformycin (8-ketodCF).

1981), cutaneous T-cell lymphoma (Grever et al., 1983, 1985), and hairy cell leukemia (Johnston et al., 1986; Kraut et al., 1986; Spiers et al., 1987). It has also been used in viral and cancer chemotherapy to prevent the deamination of adenine nucleoside analogues (e.g., ara-A) (Hershfield et al., 1983). Although the primary mechanism of action of dCF involves the inhibition of cellular adenosine deaminase (ADA), the conversion of dCF to its 5'-mono-, -di-, and -triphosphates and its subsequent incorporation into the DNA of T-lymphoblastoid cells in culture have been reported (Siaw & Coleman, 1984).

The structure of dCF, as determined by single-crystal X-ray analysis, is (8*R*)-3-(2-deoxy-β-*erythro*-pentofuranosyl)-3,6,7,8-tetrahydroimidazo[4,5-*d*][1,3]diazepin-8-ol (Woo et al., 1974). The chemical syntheses of dCF, and analogues of dCF have been reported (Baker & Putt, 1979; Chan et al., 1982; Hawkins et al., 1983; Schaumberg et al., 1985). dCF and its analogues are tight-binding transition-state inhibitors of ADA, with a K_i for dCF of 2.5×10^{-12} M (Agarwal et al., 1977). The tight-binding property is attributed to the stereochemical configuration of the hydroxyl group at C-8. For example, the chemically synthesized 8*S*-dCF and 8-ketodCF are $\sim 10^7$ times weaker inhibitors of ADA than the naturally occurring 8*R*-dCF (Schramm & Baker, 1985).

In dCF biosynthesis, the 1,3-diazepine ring, which is known to occur naturally in only dCF and analogues of dCF with different carbohydrate moieties, is derived from an adenine nucleoside or nucleotide and a one-carbon source (Hanvey et al., 1984). C-1 of D-ribose is the source of the CH_2 carbon (i.e., C-7) of dCF (Hanvey et al., 1987). Therefore, in dCF biosynthesis, the pyrimidine portion of a purine ring is expanded to form a (7-membered) 1,3-diazepine ring. These in vivo results can be explained by a mechanism in which the first reaction would be the condensation of C-1 of PRPP to N-1 of ATP, as is known to occur for histidine biosynthesis (Ames et al., 1961). In dCF biosynthesis, C-1 of PRPP would be inserted into the ring to form C-7 of dCF, whereas in histidine biosynthesis all five carbons of PRPP along with N-1 and C-2 of ATP are used to form histidine.

An intermediate in our proposed mechanism of dCF biosynthesis is 8-ketodCF (or 8-ketoCoF or one of their nucleotides) (Figure 1B). Therefore, we have investigated the possibility that 8-ketodCF and 8-ketoCoF are intermediates in the biosynthesis of dCF and CoF. In this study, we show that an enzyme partially purified from cell-free extracts of *S. antibioticus* can stereospecifically reduce 8-ketodCF and 8-ketoCoF to the naturally occurring isomer (8-*R*) of dCF and CoF, respectively. Therefore, 8-ketodCF and 8-ketoCoF, which are intermediates in the chemical synthesis of dCF and CoF, may also be biosynthetic intermediates of dCF and CoF.

Materials and Methods

Materials. [4-^{3}H]NAD$^+$ (1.0 Ci/mmol) was purchased from Amersham and D-[1-^{3}H]glucose (10 Ci/mmol) from New England Nuclear. Adenosine deaminase (ADA, adenosine aminohydrolase, EC 3.5.4.5, type I, calf mucosal), glucose-6-phosphate dehydrogenase (EC 1.1.1.49, type V, yeast) isocitrate dehydrogenase (NADP$^+$ specific, EC 1.1.1.42, type IV, porcine heart), NAD$^+$ kinase (EC 2.7.1.23, type IV, liver), and hexokinase (EC 2.7.1.1, type VI, yeast) were purchased from Sigma Chemical Co. Cellulose thin-layer sheets No. 13254 with fluorescent indicator were purchased from Eastman, and chromatography paper (3MM) was purchased from Whatman. Reverse-phase high-performance liquid chromatography (HPLC) was performed using a Waters Associates C_{18} μBondapak column (0.45 × 30 cm). Ion-exchange HPLC was performed using a Waters Associates Partisil PXS 10/25 SAX column (0.4 × 21 cm). Protein desalting was with Sephadex G-10, and protein chromatography was with Sephadex G-100 (Pharmacia). Radioactivity was determined by liquid scintillation spectrometry using a Beckman LS-100C with ACS (Amersham) as the scintillant.

Chemical Synthesis of 8-KetodCF and 8-KetoCoF. 8-KetodCF was synthesized by condensation of the 6,7-dihydroimidazo[4,5-*d*][1,3]diazepin-8(3*H*)-one with 2-deoxy-3,5-di-*O*-*p*-toluoyl-D-*erythro*-pentofuranosyl chloride, followed by separation of the anomers and deacylation (Baker & Putt, 1979; Chan et al., 1982). The chemical synthesis of 8-ketoCoF was accomplished by condensation of the 8-keto heterocycle with 2,3,5-tri-*O*-benzoyl-D-ribofuranosyl chloride and subsequent deacylation (Hawkins et al., 1983).

Growth and Maintenance of S. antibioticus Cultures. The inoculation procedure, growth conditions, maintenance, and isolation of dCF were as described (Hanvey et al., 1984, 1987).

Enzyme Isolation and Partial Purification. Each step below was at 4 °C unless stated otherwise. A 50-mL aliquot of an *S. antibioticus* culture, with a dCF concentration of ~3 μg/mL, was centrifuged at 1000*g* for 10 min. The cell pellet was resuspended in 40 mL of lysis buffer (50 mM HEPES, pH 8.0, 10% glycerol, 2 mM 2-mercaptoethanol, 5 mM EDTA) and recentrifuged. The cell pellet was resuspended in 20 mL of lysis buffer containing 2 mM of phenylmethanesulfonyl fluoride (PMSF), and the cells were disrupted by two passes through a French pressure cell (American Instruments Co.) at 16000 psi. The cell extract was centrifuged at 25000*g* for 30 min. The supernatant was stored at −70 °C in 1-mL aliquots.

To 23-mL aliquots of the cell-free extract, 5.6 g of ammonium sulfate (0–40% saturation) was added over 5 min. The solution was stirred for 30 min and then centrifuged at 12000*g* for 10 min. To 20 mL of the supernatant, 5.7 g of ammonium sulfate (40–80% saturation) was added, and the solution was treated as described above. The pellet was dissolved in 3–5 mL of lysis buffer, and the protein fraction was separated from the salts by Sephadex G-10 chromatography with a column volume 4 times the volume of the solution.

A 2.0-mL aliquot of the desalted 40–80% ammonium sulfate fraction was chromatographed by using a Sephadex G-100 column (2.5 × 40 cm) that was equilibrated with lysis buffer. The proteins were eluted from the column with lysis buffer (flow rate = 0.2 mL/min). Fractions (1 mL) were collected and assayed for reductase activity.

Protein concentrations were determined by the Bradford method (Bradford, 1976).

8-KetodCF Reductase Assay. The determination of reductase activity was at 25 °C for 15 min in a reaction (typ-

ically 20 μL) with the following composition: 50 mM HEPES buffer, pH 8.0, 1 mM NADPH, 0.5 mM 8-ketodCF or 8-ketoCoF, and desalted 40–80% ammonium sulfate protein fraction (typically 15 μL) (protein concentration = 4.5 mg/mL). The specific activity was expressed as micromoles of dCF or CoF formed per minute per milligram of protein. The reactions were terminated by the addition of 4 volumes of 95% ethanol, and, after 10 min at 4 °C, the denatured proteins were removed by centrifugation at 8000*g* for 5 min. The amount of dCF or CoF formed was routinely quantitated by an ADA assay (Hanvey et al., 1984) using an aliquot (typically 1 μL of a 20–50-fold dilution) of the supernatant.

For the positive identification of dCF and CoF as the ADA inhibitors that were formed in the enzymatic reaction, the typical 8-ketodCF reductase assays and control reactions without NADPH were increased to 1 mL and the incubation time was 2 h. After the 2-h reaction, 30 units of ADA (70 μL, ~3 nmol) was added to each, and the mixtures were incubated for 2 h at 4 °C. The protein fraction of each mixture was desalted by using a Sephadex G-10 column (1.5 × 3.5 cm) with 50 mM pH 7.5 sodium phosphate as the buffer. The proteins were denatured and then removed by heating the solutions to 90 °C for 5 min, cooling to 4 °C, and centrifuging at 8000*g* at 4 °C. Aliquots of the supernatant (200 μL) were analyzed by reverse-phase HPLC (50 mM ammonium phosphate, pH 7.0:methanol, 96:4 v/v; flow rate = 1.5 mL/min; 0.5-min fractions collected).

Enzymatic Synthesis of [4(R)-^{3}H]NADPH and [4(S)-^{3}H]NADPH. [4-^{3}H]NAD$^+$ was converted to [4-^{3}H]NADP$^+$ by using NAD$^+$ kinase as follows: 5 mM NAD$^+$, 20 μCi [4-^{3}H]NAD$^+$, 5 mM ATP, and 5 mM $MgCl_2$ were incubated in 65 mM Tris buffer, pH 7.4 (200-μL reaction) for 3.5 h at 20 °C. The reaction was terminated by the addition of 200 μL of ethanol, the proteins were removed by centrifugation, and the supernatant was taken to dryness. The [4-^{3}H]NADP$^+$ was purified by reverse-phase HPLC (10 mM ammonium phosphate, pH 7.0). The yield of [4-^{3}H]NADP$^+$ was ~9% (1.7 μCi).

[4(*R*)-^{3}H]NADPH was synthesized in a quantitative yield from [4-^{3}H]NADP$^+$ by using glucose-6-phosphate dehydrogenase. The reaction (80 μL), which contained 35 mM glucose 6-phosphate, 1 unit of glucose-6-phosphate dehydrogenase, 0.85 μCi of [4-^{3}H]NADP$^+$ (43 nmol), and 1.3 mM NADP$^+$ (total concentration of NADP$^+$ was 1.8 mM) at pH 7.0, was incubated for 15 min at 25 °C.

[4(*S*)-^{3}H]NADPH was synthesized in a quantitative yield from [4-^{3}H]NADP$^+$ by using isocitrate dehydrogenase. The reaction (80 μL), which contained 50 mM isocitrate, 2 units of isocitrate dehydrogenase, 0.3 mM $MgSO_4$, 0.85 μCi of [4-^{3}H]NADP$^+$ (43 nmol), and 1.3 mM NADP$^+$ (total concentration of NADP$^+$ was 1.8 mM), was incubated for 15 min at 25 °C. Alternatively, [4(*S*)-^{3}H]NADPH was synthesized by using D-[1-^{3}H]glucose (19 μCi), 0.1 mM D-glucose, 1 unit of hexokinase, 1 unit of glucose-6-phosphate dehydrogenase, 1 mM ATP, 0.2 mM $MgCl_2$, and 0.2 mM NADP$^+$ in a 200-μL reaction for 10 min at 25 °C.

Stereospecificity of the C-4 Hydrogen Transfer from NADPH to 8-KetodCF. In separate 8-ketodCF reductase reactions (230 μL), [4(*R*)-^{3}H]NADPH or [4(*S*)-^{3}H]NADPH (0.74 μCi, 6 nmol) was added instead of NADPH. The reactions were incubated for 1 h and terminated with 4 volumes of ethanol, and the proteins were removed by centrifugation. The supernatant was taken to dryness and then resuspended in 300 μL of 20 mM pH 7.0 ammonium phosphate buffer. Aliquots (200 μL) were analyzed by reverse-phase HPLC (50 mM ammonium phosphate, pH 7.0:methanol, 96:4 v/v; flow rate = 1.5 mL/min; 0.5-min fractions collected).

Determination of Isotope Effect. The kinetic isotope effect was calculated by using the equation

$$\frac{k_H}{k_T} = \frac{\log (1 - F)}{\log \{1 - [(F)(R_p/R_0)]\}}$$

where F is the fractional amount of conversion of substrate to product, R_p is the specific activity of the product, and R_0 is the specific activity of the substrate (Melander & Saunders, 1980).

RESULTS

The results of the incorporation and distribution of ^{3}H, ^{14}C, and ^{13}C precursors into dCF from in vivo experiments have identified adenosine and C-1 of D-ribose as the precursors for the biosynthesis of dCF (Hanvey et al., 1984, 1987). However, little is known concerning specific intermediates in the biosynthesis of dCF. Therefore, the aim of this investigation was to determine whether 8-ketodCF and 8-ketoCoF, which are intermediates in the chemical synthesis of dCF and CoF, were also intermediates in the biosynthesis of dCF and CoF.

Formation of an Inhibitor of Adenosine Deaminase by Extracts of S. antibioticus. Cell-free extracts of *S. antibioticus* were prepared when the rate of dCF formation was maximal (dCF concentration ~3–6 μg/mL). The extracts were fractionated on a Sephadex G-10 column prior to use because any NADPH and NADH added to the crude extract was rapidly oxidized. After such treatment, NADPH and NADH that were added to the protein fraction were not oxidized.

When 8-ketodCF was added to the protein fraction along with NADPH and NADH, an inhibitor of adenosine deaminase was formed as a function of time. Without the keto compound or without NADPH and NADH in the reactions no ADA inhibitor was formed. The requirement for 8-ketodCF *and* NADPH or NADH for formation of an ADA inhibitor implies that the keto compound is enzymatically reduced to form dCF. Similarly, incubation of 8-ketoCoF, NADPH, and NADH with the protein fraction produced an ADA inhibitor, presumably CoF (data not shown).

Identification of dCF and CoF as the Inhibitors Formed following the Enzymatic Reduction of 8-KetodCF and 8-KetoCoF. If the ADA inhibitors formed were dCF and CoF, the amount produced in these initial experiments was ~1 μM when 1 mM 8-ketodCF or 8-ketoCoF was used in the reaction. The efficiency of the reaction was later increased many fold (see below). To confirm that dCF and CoF were the inhibitors formed, a procedure was designed to separate the dCF or CoF from the 8-ketodCF or 8-ketoCoF. Briefly, after incubation of the protein fraction with NADPH, NADH, and either 8-ketodCF or 8-ketoCoF, a stoichiometric amount of ADA (1 mol of enzyme:1 mol of dCF or CoF) was added to the mixture to bind the putative dCF or CoF. The protein fraction was isolated free of most of the 8-keto compound by a Sephadex G-10 column, and after the proteins were denatured, the supernatant was chromatographed by using reverse-phase HPLC.

Shown in Figure 2 is a tracing of a chromatogram along with ADA inhibition of each HPLC fraction. A small peak with the same retention time as authentic dCF was present in the complete reaction when 8-ketodCF was the substrate (Figure 2A). A control reaction without NADPH and NADH, in which no ADA inhibitor was formed, did not contain this peak (Figure 2B). Furthermore, in the complete reaction, ADA inhibition was found only in the fractions that

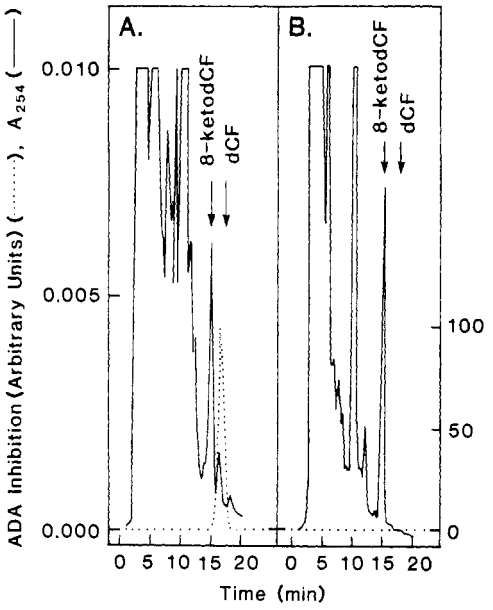

FIGURE 2: HPLC profile identifying dCF as the ADA inhibitor formed by the incubation of 8-ketodCF, NADPH, and NADH with a cell-free extract of *S. antibioticus*. In each panel, the continuous absorbance (—) and ADA inhibition of 0.5-min fractions (···) are shown. ADA inhibition is shown as arbitrary units. (A) Complete 8-ketodCF reductase reaction. (B) 8-ketodCF reductase reaction without NADPH and NADH; no ADA inhibitor was detected in this reaction prior to HPLC analysis. The arrows indicate the elution times of authentic dCF and 8-ketodCF.

would correspond to authentic dCF, and the amount of inhibition was equal to the total amount of ADA inhibition loaded onto the HPLC.

By use of the same protocol, when 8-ketoCoF was the substrate, CoF, but not dCF, was detected in the HPLC eluate (data not shown). Therefore, 8-ketodCF is reduced to dCF and 8-ketoCoF is reduced to CoF by a cell-free extract of *S. antibioticus*. The presence of *S*-dCF and *S*-CoF could not be detected by this procedure, as these compounds are only weakly bound by ADA (Schramm & Baker, 1985).

Partial Purification of the 8-KetodCF Reductase. The partial purification of the reductase was accomplished by ammonium sulfate precipitation and by Sephadex G-100 chromatography. A 40–80% ammonium sulfate fraction of the extract recovered 89% of the 8-ketodCF reductase activity, with a 3-fold increase in specific activity. Sephadex G-100 column chromatography of this fraction yielded one peak of activity (~40% recovery) with a 5–10-fold total purification. The failure to achieve a higher degree of purification and recovery after the gel filtration step was partially due to the instability of the reductase.

In order to obtain a protein fraction that was suitable for the experiments which follow, a rapid desalting of the 40–80% ammonium sulfate fraction over Sephadex G-10 was performed. This gave a concentrated protein fraction (~8 mg/mL) with high activity.

Properties of the Partially Purified 8-Keto Reductase. The rate of formation of dCF or CoF was dependent on the concentration of 8-ketodCF or 8-ketoCoF in the reaction mixture. The apparent K_m for 8-ketodCF and 8-ketoCoF were calculated as 250 and 150 μM, respectively. Also, the yield of CoF from 8-ketoCoF was ~2-fold greater than the yield of dCF from 8-ketodCF under identical conditions (Figure 3). Thus, 8-ketoCoF appears to be a better substrate for the reductase than 8-ketodCF.

Although NADH can be utilized as the reducing agent for the reductase, NADPH gives an approximately 25-fold higher

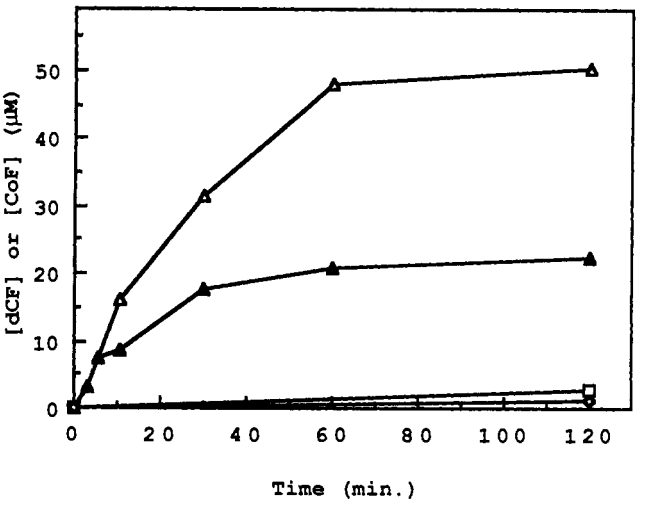

FIGURE 3: Substrate and cofactor specificity of 8-ketodCF reductase. Reactions contained the partially purified 8-ketodCF reductase, either 1 mM NADPH or 1 mM NADH, and either 0.5 mM 8-ketodCF or 0.5 mM 8-ketoCoF. Aliquots were removed at various times, and the amount of dCF or CoF was determined by ADA assay. (Open triangles) 8-ketoCoF and NADPH; (closed triangles) 8-ketodCF and NADPH; (open squares) 8-ketoCoF and NADH; (open diamonds) 8-ketodCF and NADH.

yield of dCF or CoF (Figure 3). Thus, NADPH is probably the cofactor that would be used in vivo for reduction of the 8-keto group.

The addition of ATP is not required for the enzymatic reduction of either 8-ketodCF or 8-ketoCoF, and, in fact, the addition of ATP to the reaction at a concentration of 1 mM had no effect on the reaction.

The pH optimum for the reaction was pH 8.0, and the temperature optimum was 25 °C; at 55 °C there was no reductase activity with either substrate. The rate of formation of dCF and CoF was linearly dependent on the amount of protein present in the reaction, up to 6 mg/mL.

Formation of 8-KetodCF from dCF by the Reductase. The reversibility of 8-ketodCF reductase was determined by incubating dCF and $NADP^+$ (1 mM each) with the extract and monitoring the change in absorbance at 340 nm. There was a slow, linear increase in OD_{340}, which after 1 h corresponded to approximately 10 μM NADPH (1% conversion). This increase in absorbance and thus presumably the formation of NADPH from $NADP^+$ were dependent on the presence of dCF in the reaction.

Stereospecificity of the Hydrogen Transfer from NADPH. NAD^+- and $NADP^+$-dependent dehydrogenases are classified as either A-stereospecific enzymes (which transfer the *pro-R* hydrogen from C-4 of the nicotinamide ring to the substrate) or B-stereospecific enzymes (which transfer the *pro-S* hydrogen) (Popjak, 1970). The stereospecificity of 8-ketodCF reductase was determined by the addition of either [4(*R*)-^{3}H]NADPH or [4(*S*)-^{3}H]NADPH to the reaction mixtures and subsequent HPLC analysis of the reaction products. Figure 4A, which shows a peak of ^{3}H with a retention time identical with that of dCF when [4(*S*)-^{3}H]NADPH was included in the reaction, clearly demonstrates that the *pro-S* hydrogen was transferred from NADPH to the 8-keto group to form dCF. Furthermore, Figure 4b shows that no ^{3}H was incorporated into dCF when [4(*R*)-^{3}H]NADPH was in the reaction and thus indicates that the *pro-R* hydrogen is not transferred to 8-ketodCF. Likewise, the *pro-S*, but not the *pro-R*, hydrogen was transferred from NADPH to 8-ketoCoF (parts C and D of Figure 4, respectively). Thus, 8-ketodCF reductase is a B-stereospecific enzyme.

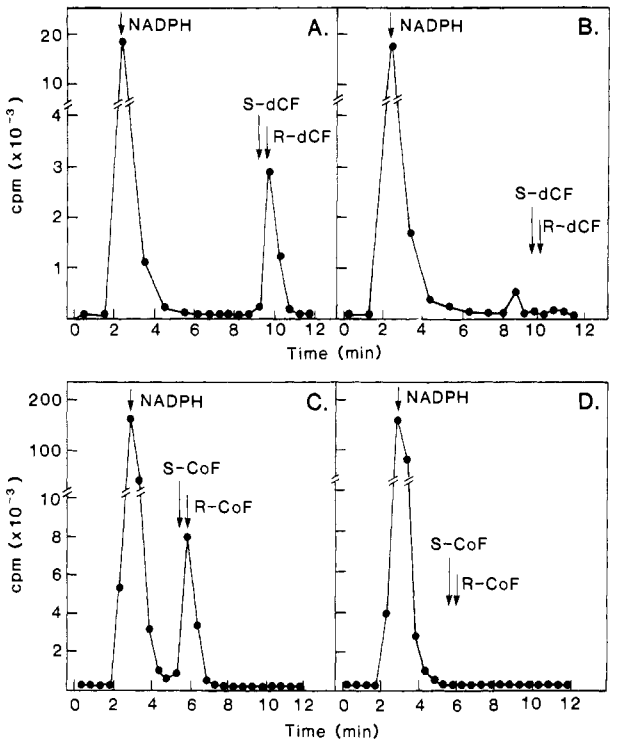

FIGURE 4: Stereospecificity of the hydrogen transfer from NADPH to 8-ketodCF and 8-ketoCoF. Shown is the amount of ^3H in 0.5-min fractions after HPLC analysis of 8-ketodCF reductase reactions containing [4(*S*)-^{3}H]NADPH and 8-ketodCF (A), [4(*R*)-^{3}H]NADPH and 8-ketodCF (B), [4(*S*)-^{3}H]NADPH and 8-ketoCoF (C), and [4(*R*)-^{3}H]NADPH and 8-ketoCoF (D). The arrows indicate the elution times of the authentic compounds.

Although the ability to identify the cofactor stereospecificity in a crude extract was significant, more importantly, these results indicate that only the *R* isomers of dCF and CoF were formed during the reduction reaction. The *S* isomers of dCF and CoF, which were resolved from the *R* isomers with the HPLC conditions used, were not detected (Figure 4).

The amount of ^3H transferred from [4(*S*)-^{3}H]NADPH to 8-ketodCF and 8-ketoCoF was significantly lower than expected on the basis of yields of the enzymatic reductions. For example, the specific activity of the [4(*S*)-^{3}H]NADPH in one experiment was 7.4 μCi/μmol, whereas the specific activity of the CoF that was isolated was 3.5 μCi/μmol. This decrease in specific activity was not due to an exchange of ^3H with water during the reduction. The 4*S* hydrogen of NADPH was probably transferred directly to 8-ketodCF and 8-ketoCoF, but an isotope effect was occurring in the reaction. On the basis of differences in the specific activities of the [4(*S*)-^{3}H]NADPH and the [^{3}H]CoF, the observed isotope effect (k_H/k_T) was 2.2, which is similar to the isotope effects reported for other enzymatic reductions (Richards, 1970).

Discussion

8-KetodCF and 8-ketoCoF have been identified as possible intermediates in the biosynthesis of dCF and CoF, respectively. These results complement our earlier in vivo studies on the biosynthesis of dCF, which revealed that adenosine and C-1 of D-ribose are the carbon–nitrogen precursors of dCF (Hanvey et al., 1984, 1987). Because the C-7 of dCF is derived directly from C-1 of D-ribose and the tetrahydrofolate one-carbon pool is not involved, a mechanism similar to the initial steps of histidine biosynthesis was proposed to explain the direct participation of D-ribose as the one-carbon donor (Figure 5) (Hanvey et al., 1987). Intermediate A of Figure 5, which is involved in the biosynthesis of histidine (Smith & Ames, 1964),

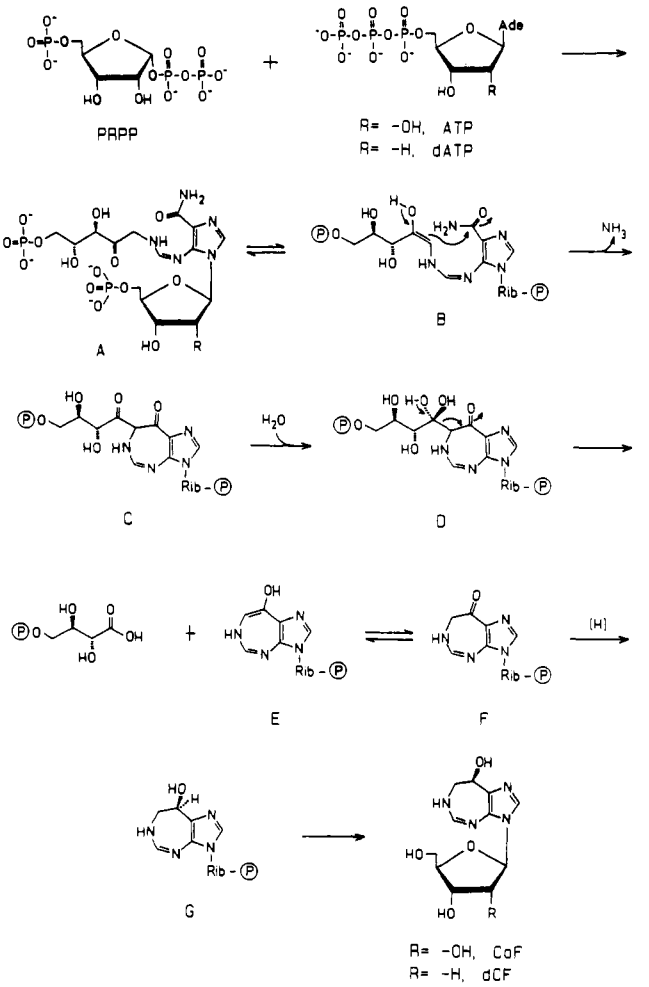

FIGURE 5: Proposed mechanism for the biosynthesis of dCF by *S. antibioticus*. The degree of phosphorylation of intermediates A–G is undetermined. The experiments described herein on intermediate F were with the nucleoside (not phosphorylated).

would be formed by condensation of PRPP at N-1 of ATP (or possibly dATP for dCF), cleavage of the ring between N-1 and C-6, and an Amadori-type rearrangement at C-1′ and C-2′ of the ribose 5-phosphate. After formation of the 1,3-diazepine ring via an aldol-like process and elimination of C-2–C-5 of what was PRPP as a tetronic acid fragment (B–D), an 8-keto analogue of dCF would remain (compound F). A reduction of the 8-keto group would form the monophosphates of dCF or CoF (G), which would be dephosphorylated to form dCF or CoF. Because the nucleosides of F (8-ketodCF or 8-ketoCoF) were chemical intermediates in the synthesis of dCF and CoF (Baker & Putt, 1979; Chan et al., 1982) and thus available, we investigated their role as possible intermediates in the biosynthesis of dCF and CoF.

In this study, it has been clearly demonstrated that a partially purified enzyme from *S. antibioticus* reduces 8-ketodCF and 8-ketoCoF to dCF and CoF, respectively. Initially, 8-ketodCF and 8-ketoCoF, at 0.5 mM, could be converted to dCF and CoF in yields of only about 0.1%. However, the percent conversion of 8-ketodCF and 8-ketoCoF, at 0.5 mM, to dCF and CoF was increased about 250-fold by using the partially purified enzyme and optimizing the buffer and reaction conditions.

That the reduction occurs at the nucleoside level indicates that the 8-keto compounds may be the penultimate intermediates in the formation of dCF and CoF. However, the effect of 5′-phosphorylation of the 8-keto compounds on their enzymatic reduction is unknown.

Although the in vivo production of CoF was approximately 30-fold lower than that of dCF in our organism (<1 μg/mL for CoF, ~20 μg/mL for dCF), both 8-keto compounds were efficiently reduced by the partially purified enzyme. The low amount of CoF excreted by our cultures could be explained by CoF being an intermediate for dCF, where any CoF produced would be rapidly reduced at the 2′-position to form dCF. Alternatively, an earlier step in the formation of dCF, perhaps the initial biosynthetic reaction, would preferentially utilize a 2′-deoxynucleoside or -deoxynucleotide. This would limit the amount of 8-ketoCoF, and thus CoF, produced by *S. antibioticus*. In either case, on the basis of our in vitro results, it is likely that substituents on the 2′-position may not have a large influence on the reduction at the C-8 position.

It is possible, although unlikely, that separate enzymes in the partially purified enzyme preparation were reducing 8-ketodCF and 8-ketoCoF. Also, this study does not prove or disprove that the enzyme which reduces 8-ketodCF and 8-ketoCoF to dCF and CoF is unique to this organism or is involved only in the production of these compounds. However, this does not diminish the implication that the 8-keto compounds are biosynthetic intermediates, as production of a secondary metabolite could utilize enzymes involved in primary metabolism.

Pyridine nucleotide-dependent enzymes catalyze a stereospecific removal of either the *pro*-R or *pro*-S hydrogen from C-4 of NADPH or NADH (Popjak, 1970). In order to determine if the reductase was a type A enzyme (*pro-R* hydrogen removed) or type B enzyme (*pro-S* hydrogen removed), the 4(*R*)-^{3}H and 4(*S*)-^{3}H isomers of NADPH were synthesized and utilized instead of NADPH as the cofactor in the assay. After isolation of the dCF or CoF by HPLC, it was found that the 4(*S*) ^{3}H, but not the 4(*R*) ^{3}H, was transferred to 8-ketodCF and 8-ketoCoF by the reductase. Therefore, the reductase is a type B enzyme. A more significant observation gained from these experiments is that 8-ketodCF and 8-ketoCoF are reduced only to the naturally occurring 8(*R*)-dCF and 8(*R*)-CoF; the 8*S* isomers of dCF and CoF were not detected. This identification was made by HPLC under conditions in which the *R* and *S* isomers of both dCF and CoF are easily separated. Our usual assay for reduction of the 8-keto compounds, which was formation of an ADA inhibitor, would not detect formation of the 8*S* isomers.

That only the naturally occurring *R* isomers of dCF and CoF are formed in vitro supports the idea that an enzyme in the biosynthetic pathway of dCF and CoF has been identified and that 8-ketodCF and 8-ketoCoF are intermediates in this process.

Registry No. 8-KetodCF, 69196-03-8; 8-ketoCoF, 90494-43-2; dCF, 70865-77-9; CoF, 11033-22-0; NADPH, 53-57-6; 8-ketodCF reductase, 114995-16-3.

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