A Highly Specific d-Hydroxyisovalerate Dehydrogenase from the Enniatin Producer Fusarium sambucinum*

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A highly specific d-hydroxyisovalerate (d-HIV) dehydrogenase, which is a key enzyme in depsipeptide synthesis, was purified to near homogeneity from the enniatin-producing fungus Fusarium sambucinum. The enzyme catalyzes the reversible reaction of 2-ketoisovalerate (2-KIV) to d-HIV. It is strictly dependent on NADPH and exhibits a high substrate specificity with respect to 2-KIV. NADH was not accepted by the enzyme. Km values for 2-KIV and NADPH were found to be 200 and 333 μM, respectively. d-HIV dehydrogenase consists of a single polypeptide chain with a molecular mass of about 53 kDa. Optimum temperature for the reduction of 2-KIV was 35 °C and for the oxidation reaction was 45 °C. The optimum pH was found to be 7 for the reduction and 8–9 for the oxidation reaction.

There are many reports about cyclodepsipeptide and peptolides which contain various hydroxy acids, such as d-HIV in enniatins, beauvericin, destruxin, and valinomycin (1–4). Enniatins are produced by several strains of the genus Fusarium and exhibit antibiotic properties due to their ionophoric activity (5). They are composed of 3 residues each of d-HIV and an N-methylated branched-chain amino acid, which are arranged in an alternative fashion (Fig. 1). Enniatins are synthesized by a multifunctional enzyme called enniatin synthetase from their primary precursors d-HIV and a branched-chain amino acid under the consumption of AdoMet and ATP (6–9). Thus the hydroxy acid is an intermediate in the biosynthetic pathway of enniatins in Fusarium. In mammals, the branched-chain amino acids leucine, isoleucine, and valine are usually transaminated to form 2-keto acids. These 2-keto acids are oxidatively decarboxylated by mitochondrial branched-chain 2-ketoacid dehydrogenase (10–12) and converted to acyl CoA. However, in some fungal species, namely the enniatin producers of the genus Fusarium, there is another pathway leading from L-Val to d-HIV via the keto acid (Fig. 2). In this paper we describe the purification of a d-HIV dehydrogenase from Fusarium sambucinum, which catalyzes the formation of d-HIV from 2-KIV.

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§ The abbreviations used are: d-HIV, d-hydroxyisovalerate; AdoMet, S-adenosyl-L-methionine; 2-KIV, 2-ketoisovalerate; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

EXPERIMENTAL PROCEDURES

Materials—Chemicals were of the highest purity commercially available. L-[^14]C]Val was purchased from Amersham Corp. Pyruvate, 2-ketobutyrate, 2-KIV, 2-ketoisovalerate, and 2-ketoisocaproate were obtained from Sigma. Radioactive 2[^14]C]KIV was prepared from radioactive L-[14]C]Val by L-amino acid oxidase and catalase (13). d-HIV was a product of Serva (Heidelberg, Germany). Enniatin synthetase was purified using the procedure described by R. Zocher et al. (6).

Growth of Organisms—F. sambucinum was maintained on FCM agar slants (3% molasses, 1% cornsteep liquor, 1.5% agar). For precultures we used chemically defined medium (FDM) as developed by Madry et al. (14). FDM contained the following ingredients per liter of distilled water: 12.5 g of glucose, 4.25 g of NaNO3, 5 g of NaCl, 2.5 g of MgSO4·7H2O, 1.36 g of KH2PO4, 0.01 g of FeSO4·7H2O, and 0.002 g of ZnSO4·7H2O. Spore suspensions of precultures were obtained by filtration of 4-day-old submerged cultures through a double layer of Cleenex cloth. Spores were inoculated in 500-ml Erlenmeyer flasks containing 100 ml of FCM liquid medium and cultivated on a rotary shaker (105 rpm, 27 °C). Cultures were harvested by suction filtration about 90 h after inoculation. The mycelial cake was washed with 0.5 M KCl in 50 mM potassium phosphate buffer and shock-frozen at –80 °C.

Mutation of the Organisms—After mutation with N-methyl-N'-nitro-N-nitrosoguanidine (NTG) for the isolation of high enniatin-producing species (14), F. sambucinum 16–4R was isolated and used for all experiments. The content of enniatin was measured spectrophotometrically using the procedure described by Audhya and Russel (15).

Enzyme Assays—The standard d-HIV dehydrogenase assay mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0.7 mM 2-KIV, 0.29 mM NADPH, and enzyme in a final volume of 0.35 ml. The reaction was initiated by the addition of substrate, and the decrease in absorbance at 340 nm was measured at 35 °C by using an UVICON 930 spectrophotometer. A molar extinction coefficient of 6.22 cm2/μmol NADPH was used for the calculation of enzyme activity. One unit was defined as the amount of enzyme catalyzing the oxidation of 1 μmol of NADPH/min under the standard assay conditions. Specific activity was expressed as units of enzyme activity/mg of protein. The reaction mixture for the assay of the reverse reaction contained 50 mM Tris/HCl buffer (pH 8.9), 2.85 mM NADP+, 5.7 mM d-HIV, and enzyme in a final volume of 0.35 ml. The increase in the rate of reduction of NADP+ due to oxidation of d-HIV was measured at 340 nm (65 °C). Enniatin synthetase activity was determined as described by Zocher et al. (6) measuring the formation of L[^14]C]Val-labeled enniatin B.

SDS-Polyacrylamide Gel Electrophoresis—SDS-polyacrylamide gel electrophoresis was done as described by Laemmli (16). Gels contained 10% acrylamide and 0.2% bisacrylamide. The relative molecular mass of the enzyme was determined from its mobility related to those of the standard proteins. Gels were silver-stained using the procedure described by Blum et al. (17).

Protein Determination—Protein concentrations were determined by a modified Bradford procedure (18) with bovine serum albumin as a standard.

Biosynthesis of Enniatin by Enniatin Synthetase and d-HIV Dehydrogenase—The reaction mixture contained 50 μl (175 μg/ml) of enniatin synthetase, 20 μl (20 μg/ml) of d-HIV dehydrogenase, 0.1 μCi of radioactive 2-KIV, 5 μl (20 mM) of NADPH, 165 μl (50 mM)

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of potassium phosphate buffer, and 10 μl of a mixture containing 0.1 M AdoMet, 1 mM MgCl₂, and 0.1 mM ATP (AdoMet:MgCl₂:ATP = 1:5:1). Reaction products were extracted with EtOAc and analyzed by TLC (ethylacetate:methanol:water = 100:5:1, v/v/v) and autoradiography (8).

**Purification of d-HIV Dehydrogenase**—All operations were carried out at 4 °C. 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 4 mM diethylythritol (buffer A) was used for all operations. The mycelial cake was lyophilized and subsequently homogenized with sand in a mortar. After the extraction of soluble proteins with 0.3 M KCl in buffer A by stirring for 40 min, the homogenate was centrifuged for 20 min at 20,000 × g. A solution of polyethyleneimine (8.5%, pH 7) was then added to the supernatant to give a final concentration of 0.2%. The precipitate was removed by centrifugation as described above. Saturated ammonium sulfate solution in buffer A was gradually added to the supernatant to a dramatic increase of specific activity (about 10-fold). S-Sepharose cation exchange chromatography, which yielded a single protein band in SDS-PAGE, was the last step of the purification procedure (Fig. 4, lane C).

**Molecular Mass Determinations**—Determination of molecular mass of the denatured enzyme is shown in Fig. 4. SDS-PAGE exhibited that d-HIV dehydrogenase migrates as a single protein band with a molecular mass of about 53 kDa compared with standard proteins (β-galactosidase, 116 kDa; phosphorylase G, 97.4 kDa; bovine serum albumin, 66 kDa; egg albumin, 45 kDa; carbonic anhydrase, 29 kDa). Molecular mass determinations of the native enzyme were carried out by means of size exclusion chromatography using a Sephadex G-150 column, which was previously calibrated with standard proteins (aldolase, 150 kDa; albumin, 66 kDa; carbonic anhydrase, 29 kDa) (29). The elution volume of d-HIV dehydrogenase indicated a molecular mass of 53.7 kDa for this enzyme assuming a globular structure (Fig. 5). The fact that both methods give similar molecular masses for d-HIV dehydrogenase suggests that this enzyme is composed of a single polypeptide chain.

**Kinetic Measurements**—Various substrate analogues of 2-ketoisovaleric acid including pyruvate, 2-ketobutyrate, 2-ketovalerate, 2-ketocapronate, 2-ketoisocapronate, 2-keto-3-methyl-n-valerate, and 2-ketooctanoate were used to investigate the substrate specificity of d-HIV dehydrogenase. Kinetic values were estimated from the double-reciprocal plots shown.
FIG. 4. SDS-gel electrophoresis of protein samples from different purification steps of D-HIV dehydrogenase. Lane A, 35–45% saturated (NH₄)₂SO₄ precipitation (48 µg); lane B, pooled active fractions of AcA 44 gel chromatography (20 µg); lane C, purified D-HIV dehydrogenase of S-Sepharose cation exchange chromatography (2 µg).

Fig. 5. Molecular mass of D-HIV dehydrogenase determined by Sephadex G-150 size exclusion gel chromatography.

in Fig. 6 by plotting the reciprocal concentrations of the second substrate versus the corresponding intercepts of the 1/ν axis (not shown). The enzyme exhibited high substrate specificity with respect to 2-ketoisovalerate (Kₘ = 200 µM) and 2-keto-3-methyl-n-valerate (Kₘ = 400 µM). The homologues compounds 2-ketovalerate, 2-ketoisocapronate, and 2-ketocaproate were shown to be poor substrates (Kₘ = 4.2, 5, and 4.4 mM, respectively). The enzyme was specific for NADPH as a coenzyme (Kₘ = 333 µM), which could not be replaced by NADH. The Kₘ values for the reverse reaction were found to be 9 mM for D-HIV and 350 mM for NADPH*.

The reductive reaction was inhibited competitively by the end products, D-HIV and NADPH*.

Optimal Temperature and pH of D-HIV Dehydrogenase—For determination of the optimal temperature of D-HIV dehydrogenase reaction, mixtures containing enzyme and NADPH were preincubated at various temperatures for 1 min. The reaction was started by addition of 2-KIV. An optimum of 35 °C was found for the reductive and 45 °C for the oxidative reaction (not shown).

The effect of pH on the activity of the enzyme was determined over a wide range between pH 3.7 and 10.5 using acetate, phosphate, and Tris buffer. A sharp optimum was observed for the reductive reaction at pH 7 and a plateau-like optimum between pH 8 and 9 for the oxidative reaction.

Formation of Enniatin by a Coupled Reaction of Enniatin Synthetase and D-HIV Dehydrogenase—To demonstrate that D-HIV dehydrogenase indeed produces D-HIV, we performed the experiment described in Fig. 7. As can be seen in lane C of TLC enniatin is formed in the presence of enniatin synthetase and D-HIV dehydrogenase from ⁴C-labeled 2-KIV, NADPH, AdoMet, and L-Val. In the absence of NADPH no product formation occurred (lane D).

Discussion

We have purified a highly specific D-2-hydroxyxacylic acid dehydrogenase from the depsipeptide-producing fungus F. sambucinum. This enzyme, which plays a key role in enniatin biosynthesis, catalyzes the reversible reaction of 2-KIV to D-HIV using NADPH as a cofactor which cannot be replaced by NADH.

To our knowledge this is the first report about the purification and characterization of a hydroxycarboxylic acid dehydrogenase with high substrate specificity for 2-KIV.

As reported previously by Billich and Zocher (8), enniatin synthetase in F. scirpi behaved like a constitutive protein of primary metabolism and it is also present in the stationary phase of growth. This seems to be the case with D-HIV dehydrogenase, too, as can be seen in Fig. 3. The growth curve of F. sambucinum showed that maximal activity of soluble D-HIV dehydrogenase in cell-free extracts did not coincide with that of enniatin synthetase during the fermentation. The reason for this phenomenon is unknown. The increase of the soluble D-HIV dehydrogenase activity in the end phase of enniatin production may be explained by the assumption that D-HIV dehydrogenase was membrane-bound during the production phase and is released in the stationary phase as a soluble enzyme. Similar behavior has been observed in the case of bacitracin synthetase in Bacillus licheniformis (20). If one considers the concentration of D-HIV dehydrogenase and enniatin synthetase in crude extracts of the fungus it is noteworthy that the latter enzyme is present in much higher amounts (6) compared with D-HIV dehydrogenase described in this paper. This discrepancy can be explained by the different turnover numbers of both enzymes, which have been calculated to be 7890/s in the case of D-HIV dehydrogenase and 0.1–0.5 in the case of enniatin synthetase.

NAD(P)H-dependent carbonyl oxidoreductases are valuable catalysts for the gaining of sterosepecific isomers of 2-hydroxyxacylic acid. Stereosepecific D- and L-lactic acid dehydrogenases are well known from microorganisms and L-lactate dehydrogenase from mammalian tissues (21, 22). L-Lactate dehydrogenase catalyzes predominantly the reduction of pyruvate to lactate among the various 2-keto acids. Pyruvate showed the highest V_max/K_m and other 2-keto acids, e.g. 2-KIV and 2-ketoisocapronate were poor substrates. Recently new NAD(P)-dependent stereospecific D- and L-hydroxysa- capronate dehydrogenases were reported from Lactobacillus species, which preferentially reduced 2-ketoisocapronate to D- and L-hydroxyisocapronate (23, 24). However, these enzymes exhibited low substrate affinity with respect to 2-KIV. D-HIV dehydrogenase differs from other NADPH-dependent oxidoreductases with broad substrate specificity by its high affinity for 2-KIV and with a lower extent for that of 2-keto-3-methyl-n-valerate, which exhibits a K_m value about 2 times higher.
FIG. 6. Kinetic plots for 2-KIV: NADPH. a, $1/v$ versus $1/[2\text{-KIV}]$ at various concentrations of NADPH; b, $1/v$ versus $1/[\text{NADPH}]$ at various concentrations of 2-KIV.

FIG. 7. Autoradiography of enniatin B synthesized by enniatin synthetase and D-HIV dehydrogenase with 2-KIV as a substrate. In the experiments shown in lane C and D, D-HIV (normal substrate of enniatin synthetase) was substituted with 2-[14C]KIV. All reaction mixtures contained AdoMet, ATP, and MgCl$_2$. Overall procedure is described under “Experimental Procedures.” Lane A, standard enniatin B synthesized by enniatin synthetase with L-[14C]Val as a radioactive marker and D-HIV as a substrate; lane B, enniatin B synthesized by enniatin synthetase and d-HIV dehydrogenase with 2-[14C]KIV as a radioactive marker and NADPH; lane D, enniatin synthetase and d-HIV dehydrogenase with 2-[14C]KIV (without NADPH).

than that of 2-KIV. These findings indicate that obviously the branching CH$_3$- group in the $\beta$-position is essential for the substrate to enter the binding site of the enzyme. The high specificity of d-HIV dehydrogenase may also explain the fact that d-HIV is the exclusive hydroxy acid component in enniatins isolated from Fusaria (6).

REFERENCES