The B Form of Dihydroorotate Dehydrogenase from *Lactococcus lactis* Consists of Two Different Subunits, Encoded by the *pyrDb* and *pyrK* Genes, and Contains FMN, FAD, and [FeS] Redox Centers*

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The B form of dihydroorotate dehydrogenase from *Lactococcus lactis* (DHOdehase B) is encoded by the *pyrDb* gene. However, recent genetic evidence has revealed that a co-transcribed gene, *pyrK*, is needed to achieve the proper physiological function of the enzyme. We have purified DHOdehase B from two strains of *Escherichia coli*, which harbored either the *pyrDb* gene or both the *pyrDb* and the *pyrK* genes of *L. lactis* on multicopy plasmids. The enzyme encoded by *pyrDb* alone (herein called the δ-enzyme) was a bright yellow, dimeric protein that contained one molecule of tightly bound FMN per subunit. The δ-enzyme exhibited dihydroorotate dehydrogenase activity with dichloroindophenol, potassium hexacyanoferrate(III), and molecular oxygen as electron acceptors but could not use NAD⁺. The DHOdehase B purified from the *E. coli* strain that carried both the *pyrDb* and *pyrK* genes on a multicopy plasmid (herein called the ζ-enzyme) was quite different, since it was formed as a complex of equal amounts of the two polypeptides, i.e. two PyrDB and two PyrK subunits. The ζ-enzyme was orange-brown and contained 2 mol of FAD, 2 mol of FMN, and 2 mol of [2Fe-2S] redox clusters per mol of native protein as tightly bound prosthetic groups. The ζ-enzyme was able to use NAD⁺ as well as dichloroindophenol, potassium hexacyanoferrate(III), and to some extent molecular oxygen as electron acceptors for the conversion of dihydroorotate to orotate, and it was a considerably more efficient catalyst than the purified δ-enzyme. Based on these results and on analysis of published sequences, we propose that the architecture of the ζ-enzyme is representative for the dihydroorotate dehydrogenases from Gram-positive bacteria.

Dihydroorotate dehydrogenase catalyzes the fourth chemical reaction in the biosynthesis of UMP, which is oxidation of 5,6-dihydroorotate to orotate. Genes encoding this enzyme have been cloned and sequenced from a variety of organisms. The milk-fermenting bacterium *Lactococcus lactis* is the only organism so far known to contain two dihydroorotate dehydrogenases. They have been termed dihydroorotate dehydrogenase A (DHOdehase A)¹ and dihydroorotate dehydrogenase B (DHOdehase B) and are encoded by the *pyrDa* and *pyrDb* genes, respectively (Andersen et al., 1994). Both enzymes are able to function in pyrimidine biosynthesis, since both of the genes must be inactivated by mutation in order to impose a pyrimidine requirement on *L. lactis* and since either of the two genes is able to correct the pyrimidine requirement of a *pyrD* deletion strain of *Escherichia coli* (Andersen et al., 1994). The polypeptides encoded by the *pyrDa* and *pyrDb* genes both consist of 311 amino acid residues, and the predicted amino acid sequences are 30% identical with each other. However, the sequence of DHOdehase A shows 71% amino acid identity with the sequence of the cytosolic dihydroorotate dehydrogenase from bakers’ yeast, while the sequence of DHOdehase B shows 60–70% amino acid identity with the deduced amino acid sequence of dihydroorotate dehydrogenases from *B. subtilis* and several other Gram-positive bacteria (Andersen et al., 1994; Nielsen et al., 1996).

We have initiated a study of these two dihydroorotate dehydrogenases from *L. lactis*, with the aim of comparing their functional and structural properties with the properties of the enzymes from *E. coli* (Larsen and Jensen, 1985) and other organisms. We began by purifying the two lactococcal enzymes from strains of *E. coli* that carried either *pyrDa* or *pyrDb* cloned on multicopy plasmids. DHOdehase A was a stable enzyme and proved to be a dimeric protein, containing one molecule of FMN per subunit. It was an efficient catalyst that could use dichloroindophenol, potassium hexacyanoferrate(III), or, to a lower extent, molecular oxygen as an acceptor of the reducing equivalents from dihydroorotate (Nielsen et al., 1996). However, the DHOdehase B encoded by the *pyrDb* gene turned out to be an unstable and inefficient enzyme, although it could be produced in substantial quantities in *E. coli*. We considered the possibility that this enzyme might require unusual electron acceptors or unusual incubation conditions for optimal function. While we were searching for such assay conditions, it appeared as a result of genetic studies that the activity of DHOdehase B in *L. lactis* was dependent on the integrity of a neighboring open reading frame, now termed *pyrK*, which is co-transcribed with *pyrDb* (Andersen et al., 1996). Therefore, we introduced the *pyrK* gene into our expression vector, which already carried *pyrDb*, and purified DHOdehase B from a strain of *E. coli* harboring this plasmid. The resulting enzyme was dramatically different from the enzyme encoded by the *pyrDb* gene alone, as it appeared to be a stable stoichiometric complex of PyrDB and PyrK polypeptides. Furthermore, the polypeptide chain encoded by *pyrDb*; PyrK, polypeptide chain encoded by *pyrK*; δ-enzyme, dimeric dihydroorotate dehydrogenase B containing only PyrDB subunits; ζ-enzyme, tetrameric dihydroorotate dehydrogenase B built of both PyrDB and PyrK subunits; DCIP, 2,6-dichloroindophenol; PCR, polymerase chain reaction.

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¹ The abbreviations used are: DHOdehase A and DHOdehase B, the A and B forms of dihydroorotate dehydrogenase from *L. lactis*; PyrDB, (DHOdehase B) and are encoded by the *pyrDa* and *pyrDb* genes, respectively (Andersen et al., 1994). Both enzymes are able to function in pyrimidine biosynthesis, since both of the genes must be inactivated by mutation in order to impose a pyrimidine requirement on *L. lactis* and since either of the two genes is able to correct the pyrimidine requirement of a *pyrD* deletion strain of *Escherichia coli* (Andersen et al., 1994). The polypeptides encoded by the *pyrDa* and *pyrDb* genes both consist of 311 amino acid residues, and the predicted amino acid sequences are 30% identical with each other. However, the sequence of DHOdehase A shows 71% amino acid identity with the sequence of the cytosolic dihydroorotate dehydrogenase from bakers’ yeast, while the sequence of DHOdehase B shows 60–70% amino acid identity with the deduced amino acid sequence of dihydroorotate dehydrogenases from *B. subtilis* and several other Gram-positive bacteria (Andersen et al., 1994; Nielsen et al., 1996).

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DHOdehase B of L. lactis

protein had acquired the ability to use NAD+ as a co-substrate for the oxidation of dihydroorotate.

For the sake of simplicity we have named the native enzyme encoded only by the pyrDb gene the &delta;-enzyme, and the enzyme encoded by pyrDb and pyrK was called the &delta;K-enzyme.

EXPERIMENTAL PROCEDURES

Materials—Restriction endonucleases, T4 DNA ligase, and Deep Vent (exo-) DNA polymerase were bought from either New England Biolabs or Boehringer Mannheim and used as recommended by the manufacturers. The Sequenase 2.0 kit was from U.S. Biochemical Corp. Diethylaminoethyl-cellulose (DE52) was from Whatman BioSystems Ltd. (Maidstone, United Kingdom), hydroxylapatite (Bio-Gel) was from Bio-Rad, the sonifier from Branson. The pyrK gene from plasmid pFN3, which carries both the pyrDb and pyrK genes of L. lactis, was used in this study. The cells were grown to stationary phase at 37 °C with vigorous aeration in LB broth medium (Miller, 1972) supplemented with 0.1 g/liter ampicillin. The synthesis of DHOdehase B was induced by the addition of 0.75 mM isopropyl-β-D-thiogalactoside when the optical density (A660) of the culture was 1.0. For plasmid pFN2, growth was continued for 24 h until the culture had been stationary for several hours, while the cultures containing pFN3 were harvested 3.5 h after induction because the PyrK polypeptide was slowly degraded upon prolonged incubation in the stationary phase. The cells were harvested by centrifugation for 20 min at 6000 rpm using a GS-3 rotor in a refrigerated Sorvall centrifuge, washed with 0.9% NaCl, and kept frozen at −20 °C.

The &delta;K-Enzyme—To purify the &delta;K-enzyme, frozen cell pellets from 2.5-liter stationary cultures of S.6645/pFN2 were suspended in 75 ml of ice-cold Buffer A and disrupted by ultrasonic treatment using a Branson sonifier for 15 × 0.5 min, interrupted by cooling in an ice bath for 1.5 min between cycles of sonication. Cell debris was removed by centrifugation for 20 min at 12 000 rpm in a Sorvall SS-34 rotor. 1⁄10 volume of a 10% solution of streptomycin sulfate was added to the supernatant. After stirring for 15 min, the precipitate was removed by centrifugation at 10 000 rpm for 30 min in a Sorvall SS-34 rotor. The supernatant was dialyzed as described above. The supernatant was dialyzed on a 25-ml column of blue dextran Sepharose. The column was first washed with 400 ml of Buffer A and then eluted with a 100-ml linear gradient of 0 to 0.5 M NaCl in Buffer A. Fractions with most DHOdehase activity were pooled, desalted on a Micro Ultrafiltration system (Amicon). Glycerol was added to 50%, and the enzyme was stored at −20 °C.

The purification procedure is summarized in Table 1, and an SDS-PAGE analysis of the enzyme product is shown in Fig. 2.

The &delta;K-enzyme—In order to purify the &delta;K-enzyme, frozen cell pellet from a 10-liter culture of S.6645/pFN3 was suspended in 75 ml of Buffer A and disrupted by sonication as described above. Cell debris was removed by centrifugation for 20 min at 12 000 rpm in a Sorvall SS-34 rotor. 1⁄10 volume of a 10% solution of streptomycin sulfate was added to the supernatant. After stirring for 15 min, the precipitate was removed by centrifugation as described above. The supernatant was dialyzed on a 25-ml column of blue dextran Sepharose. The column was first washed with 400 ml of Buffer A and then eluted with a 100-ml linear gradient from 0 to 1.0 M NaCl in Buffer A. Fractions with most DHOdehase activity were pooled, desalted on a Micro Ultrafiltration system (Amicon). Glycerol was added to 50%, and the enzyme was stored at −20 °C.

Assays of Dihydroorotate Dehydrogenase Activity—In the standard assay for dihydroorotate dehydrogenase activity, the oxidation of dihydroorotate was coupled to the reduction of the synthetic quinone dichloroindophenol (DCIP). The reduction of 1 μmol of DCIP causes a decrease in the absorbance at 600 nm, ε = 20 × 10³ M⁻¹ cm⁻¹ (Karabin, 1978). The spectra were recorded in a Zeiss Spectro S10 diode-array photometer. The standard assay mixture contained 0.1 M Tris-HCl, pH 8.0, 0.1 M KCN, 1 mM dihydroorotate, and 1 μM of the assay temperature was 37 °C. One unit of enzyme activity is defined as the amount of enzyme that produces 1 μmol of orotate/min under these conditions. In assays with different electron acceptors, we used the absorption at 295 nm to obtain a quantitative measure of the production of orotate (ε = 3.67 × 10³ M⁻¹ cm⁻¹).

Growth of Cells for Purification of DHOdehase B—Two forms of DHOdehase B were purified from strain S.6645, transformed either with pFN2, which carried the pyrDb gene, or with plasmid pFN3, which carries both the pyrDb and pyrK genes of L. lactis.
FIG. 1. Structure of the expression vectors pFN2, pFN3, and pFN4. Transcription of the cloned genes is driven by the very strong P<sub>Almalac</sub> promoter, which is a synthetic derivative of the early A1 promoter of phage T7 containing two binding sites for the lac repressor.Expression of cloned genes is kept repressed by the lacI<sup>+</sup> repressor until induction with isopropyl-β-D-thiogalactoside. cat, gene for chloramphenicol acetyltransferase; bla, gene for β-lactamase; λ<sub>ε</sub>, transcription terminator; pyrDb, gene encoding the PyrDB polypeptide (deduced mass = 28.6 kDa); pyrK, gene encoding the PyrK polypeptide (deduced mass = 32.8 kDa).

Determination of Flavin Content—The flavin was released from aliquots of the enzymes by treating with 0.25 M formic acid and analyzed by chromatography on poly(ethyleneimine)-impregnated cellulose thin layer plates together with authentic FMN (R<sub>p</sub> = 0.35) and FAD (R<sub>p</sub> = 0.17) as described by Larsen and Jensen (1985). In addition, the flavin was extracted from the δ-enzyme by treatment with 4% ammonium sulfate in 75% methanol as described by Alexen and Handler (1967). After pelleting the protein part of the enzyme by centrifugation, the spectrum of the supernatant was recorded and compared with the spectra of authentic FMN, FAD, and mixtures of the two flavin compounds, dissolved in 4% ammonium sulfate, 75% methanol.

Determination of Iron Content—Aliquots of the δ-enzyme (800 μl containing 1–12 nmol of enzyme in 5 mM sodium phosphate, pH 6.0) were mixed with 100 μl of 8 M HCl and incubated for 10 min at 0 °C. Protein was precipitated by the addition of 100 μl of 80% trichloroacetic acid for 10 min, and the solution was cleared by centrifugation. 200 μl of 75% ammonium acetate was added to 800 μl of the supernatant to adjust the pH to 4.5. Subsequently, 80 μl of 10% hydroxylamine hydrochloride and 80 μl of 4 mM tripyridyl-s-triazine were added, and the mixtures were incubated for 10 min. The amount of iron was quantified by measuring the absorption at 593 nm as described by Fischer and Price (1964). One nmol of Fe gave an absorption A<sub>593</sub> = 0.015.

Determination of Acid-labile Sulfide—Aliquots of enzyme (320 μl) were treated with 2.6% Zn(CH<sub>3</sub>COO)<sub>2</sub> and 0.75% NaOH for 2 h; 100 μl of 0.1% N,N-dimethyl-p-phenylenediamine, dissolved 5 M HCl, and 40 μl of 11.5 mM FeCl<sub>3</sub> in 0.6 M HCl were then added, and the solution was mixed by shaking for 1 min. Finally, 320 μl of water was added, and the sample was cleared by centrifugation. The acid-labile sulfide was quantified by measuring A<sub>593</sub> as described by King and Morris (1964). One nmol of S<sup>2-</sup> gave an absorption A<sub>593</sub> = 0.032.

Kinetic Analyses—Saturation curves from kinetic experiments were fitted to the Michaelis-Menten equation using the BIOSOFT program Ultrafit for the Macintosh.

RESULTS

Production of Dihydroorotate Dehydrogenase B—The expression vectors used for production of DHOdehase B in E. coli are described in Fig. 1. All three plasmids are derivatives of pUHE23–2 and contain the strong LacI-controlled P<sub>Almalac</sub> promoter to drive transcription of the cloned genes. Plasmid pFN2 contains only the pyrDb gene, which we initially thought would contain all coding information for DHOdehase B of L. lactis (Andersen et al., 1994). Plasmid pFN3 contains both pyrDb and pyrK, but the two genes are inserted in opposite order relative to the order by which they are transcribed from the chromosome of L. lactis. Plasmid pFN4 carries only the pyrK gene. The plasmids, pFN2 and pFN3, were able to complement the pyrimidine requirement of the E. coli strain S<sub>c</sub>6645, which is deleted for the pyrD gene, but pFN4 was not. In order to use S<sub>c</sub>6645, transformed with pFN2 or pFN3, for production of DHOdehase, it was important to grow the cultures to a considerable density while the strong P<sub>Almalac</sub> promoter was kept repressed, since growth terminated approximately one generation after induction of promoter activity by the addition of isopropyl-β-D-thiogalactoside.

Purification of the δ-Enzyme, Encoded by the pyrDb Gene on pFN2—The purification procedure for DHOdehase B encoded by pyrDb is described under “Experimental Procedures” and summarized in Table I. The enzyme could be produced in substantial amounts in an electrophoretically homogeneous form (Fig. 2). However, the enzyme was unstable, and the specific activity decreased slightly in the last steps of purification (Table I). In earlier versions of the purification, the fall in specific activity during purification was even more dramatic. The half-life of the δ-enzyme was about 45 s under assay conditions at 37 °C and was 4 min when the assays were performed at 25 °C. If a solution of the δ-enzyme in the purification buffer was left at room temperature overnight, no dihydroorotate dehydrogenase activity remained.

Purification of the δ-Enzyme, Encoded by the pyrDb and pyrK Genes on pFN3—The purification procedure for the δ-enzyme is described under “Experimental Procedures” and summarized in Table II. The resulting enzyme contained equal amounts of PyrDB and PyrK polypeptides (Fig. 2). These two polypeptides seemed to form a very stable complex with each other, since they have resisted separation over many steps of column chromatography and since they migrated as a single protein during electrophoresis in a nondenaturing agarose gel with mobility very different from the mobility of the δ-enzyme, which contained only the PyrDB subunits (Fig. 3). The complex
The activity of the \( \delta \)-enzyme was assayed without problems at 37 °C, and approximately 75% of the activity remained when the protein was incubated in the purification buffer for 20 min at 55 °C.

**Molecular Masses and Subunit Composition**—The two forms of DHO dehase B were subjected to gel filtration chromatography on a Superose 6 HR 10/30 column (Pharmacia) together with standard marker proteins. Fractions were collected and analyzed by measurements of enzyme activity and by SDS-gel electrophoresis. The \( \delta \)-enzyme eluted from the column together with bovine serum albumin, molecular mass 64–66 kDa. This gel filtration behavior indicated that the protein is a homodimer consisting of two PyrDB subunits, since the molecular mass of the subunit is 33 kDa. On the other hand, the native \( \delta \)-enzyme eluted at a position corresponding to a protein with a molecular mass of 130 kDa. This indicated that the \( \delta \)-

**TABLE I**

| Purification step         | Volume | Total activity | Total protein | Specific activity | Yield | Purification |
|---------------------------|--------|----------------|---------------|------------------|-------|--------------|
| Crude extract             | 50     | 1645           | 2136          | 0.77             | 100   | 1.0          |
| Streptomycin supernatant  | 44     | 1452           | 1126          | 1.3              | 88    | 2.0          |
| Matrex Red A              | 22     | 825            | 121           | 6.8              | 50    | 8.8          |
| DE-52                     | 54     | 621            | 92            | 6.7              | 37    | 8.7          |
| Blue Sepharose            | 78     | 312            | 54            | 5.7              | 19    | 7.4          |

**TABLE II**

| Purification step         | Volume | Total activity | Total protein | Specific activity | Yield | Purification |
|---------------------------|--------|----------------|---------------|------------------|-------|--------------|
| Crude extract             | 75     | 2734           | 3408          | 0.8              | 100   | 1            |
| Streptomycin supernatant  | 84     | 2511           | 1638          | 2                | 92    | 2            |
| DE52                      | 64     | 1984           | 249           | 8                | 73    | 10           |
| Hydroxyl apatite          | 64     | 1728           | 115           | 15               | 63    | 19           |
| Matrex Red A              | 64     | 1043           | 30            | 34               | 38    | 43           |
| Concentrated Matrex Red A| 6.3    | 663            | 19            | 34a              | 24    | 43           |

\( a \) The amount of protein in the samples were determined by the Lowry procedure (Lowry et al., 1951). According to a determination of the content of amino acid in acid hydrolysates of the protein, the Lowry procedure overestimated the absolute protein concentration by 41%. The specific activity of the purified \( \delta \)-enzyme is therefore 48 units/mg.
ally have an absorption coefficient. Tris (40 mM), sodium acetate (20 mM) and NaEDTA (1 mM) adjusted to pH 8 with acetic acid. The agarose gel (1%) in a buffer consisting of Plus and minus signs indicate the electrodes. The gel was fixed with 10% acetic acid, dried, and stained with Coomassie Brilliant Blue G-250 enzyme is a tetramer composed of two PyrDb subunits (33 kDa) and two PyrK subunits (29 kDa).

**Spectral Properties and Cofactor Content of the Two Enzymes**—The purified δ-enzyme was bright yellow, with a trace of green, and it showed an absorption spectrum typical for an oxidized flavoprotein with absorption maxima at 450 and 375 nm (Fig. 4). The absorbance at 450 nm was 0.29 per mg of protein, determined by the Lowry procedure (Lowry et al., 1951). This value indicated that the δ-enzyme contains 0.9 mol of flavin/mol of subunit (Mₚ = 33,000), since flavoproteins usually have an absorption coefficient A₄₅₀ of about 11 μm⁻¹ cm⁻¹ (Untuch-Grau et al., 1982). The flavin was released from the protein by treatment with 0.33 M formic acid and found to co-migrate with authentic FMN by thin layer chromatography, while it migrated twice as fast as FAD.

The complex δκ-enzyme was orange-brown instead of yellow, and the characteristic flavin peaks at 377 and 452 nm in the absorption spectrum (Fig. 4) were superimposed onto a broad range of absorption extending from 300 to beyond 600 nm. Upon treatment with formic acid, both FMN and FAD were released from the enzyme. The two flavin compounds were suspected under UV light. The flavins were also extracted from the enzyme with 4% ammonium sulfate and 75% methanol, leaving a yellow supernatant and a brownish protein pellet. The absorbance of the supernatant at 448 nm indicated that one mol of native δκ-enzyme contains 4.1 ± 0.1 mol of flavin. The ratio between the absorption at 268 nm and the absorption at 448 nm was compared with the similar ratios of absorbances of mixtures of authentic FMN and FAD. This analysis revealed that the enzyme contains approximately 40% FMN and 60% FAD. Based on these results, we propose that the δκ-enzyme contains 2 mol of FAD and 2 mol of FMN per mol of native tetrameric enzyme.

The absorption spectrum in Fig. 4, as well as the color of the protein, indicated that the enzyme contains iron as well as the flavins, and since it also developed a characteristic smell of sulfide when it was treated with sulfuric acid, we suspected that the protein contained iron-sulfur redox centers. The iron content was quantified by the method of Fischer and Price (1964) and a value of 3.6 ± 0.3 mol of Fe/mol of native δκ-enzyme was found. Furthermore, we found 3.2 ± 0.3 mol of acid-labile sulfide/mol of enzyme by using the method of King and Morris (1964). These iron and sulfur analyses were performed more than six times using two different preparations of the enzyme, and since the method of King and Morris (1964) usually underestimates the true content of sulfur, the data suggest strongly that one mol of native δκ-enzyme contains two mol of [2Fe-2S] redox centers. The iron-sulfur clusters are likely to be bound to the sulfhydryl-rich stretches of amino acid residues near the carboxyl termini of the PyrK subunits (Fig. 5).

**Catalytic Properties and Specificity**—Both forms of DHOdehase B displayed optimal activity around pH 8 when assayed with DCIP as electron acceptor and dihydroorotate as substrate (Fig. 6). The specificity of the enzyme reactions at pH 8 is shown in Table III. It appears that both forms of DHOdehase B could use dichloroindophenol, potassium hexacyanoferrate(III), and to a lower extent also molecular oxygen as electron acceptors for the conversion of dihydroorotate to orotate, whereas only the δκ-enzyme was able to use NAD⁺. When assayed under standard conditions at pH 8, using 50 μM DCIP as an electron acceptor, the apparent Kₘ for orotate was 28 ± 2 μM for the δκ-enzyme and 949 ± 48 μM for the δ-enzyme, while the apparent Vₘₐₓ was 3-fold higher for the δκ-enzyme.
shown is residue 220 in the PyrK protein of L. lactis which are able to reduce all natural pyrimidine bases. L. lactis logical form of DHO dehase B in L. lactis buffer consisting of Tris (50 mM), NaH2PO4 (50 mM) adjusted to the pH 6.5, and K3[Fe(CN)6] (0.45 mM) were also found to contain FMN (Hines and Johnson, 1985) and the enzymes from M. lysodeikticus and Enterococcus faecalis (Nielsen et al., 1996) and since an orf with very high similarity to pyrK is found immediately upstream of the pyrD gene in these bacteria (Andersen et al., 1996; Ghim et al., 1994; Li et al., 1995; Quinn et al., 1991). Moreover, it was shown in the case of B. subtilis that disruption of this orf, now termed pyrDII, imposes a partial pyrimidine requirement on the bacterium and strongly lowers the activity of dihydroorotat dehydrogenase in cell-free extracts (Kähler and Switzer, 1996).

The behavior of the δ-enzyme during SDS-gel electrophoresis and gel filtration indicated strongly that it consists of two PyrDB and two PyrK subunits. Spectrophotometric and chromatographic determinations showed that the native enzyme contains 2 mol of FAD and 2 mol of FMN. In addition, the iron and sulfide analyses indicated strongly that the tetrameric δ-enzyme contains 2 mol of [2Fe-2S] redox centers. The ability to bind FMN must reside in the PyrDB subunit, while the ability to bind FAD and the [2Fe-2S] clusters as prosthetic groups seems to be linked to the PyrK subunit in the complex, since the protein encoded by the pyrD gene alone, i.e. the δ-enzyme, is a functional dimeric dihydroorotat dehydrogenase that only contains FMN. Furthermore, the capability to use NAD+ as an electron acceptor is linked to the presence of the PyrK subunit in the complex, since the δ-enzyme is unable to function with NAD+ as a substrate.

The δ-enzyme resembles several other dihydroorotat dehydrogenases, e.g. dihydroorotat dehydrogenase A from L. lactis (Nielsen et al., 1996), and the enzymes from E. coli (Larsen and Jensen, 1985) and the two protozoans, Crithidia fasciculata and Trypanosoma brucei (Fascal et al., 1983). These enzymes are all dimeric dihydroorotat dehydrogenases with one FMN per subunit, and they are unable to use NAD+ as an electron acceptor. The dihydroorotat dehydrogenases purified from bovine liver mitochondria and the mitochondria of Neurospora crassa were also found to contain FMN (Hines and Johnson, 1989; Hines et al., 1986; Miller, 1975; Miller and Adams, 1971), whereas flavin could not be detected in significant quantities in

### Table III

| Electron acceptor | δ-Enzyme Activity | δ-Enzyme Activity |
|-------------------|-------------------|-------------------|
| DCIP (0.05 mM)    | 48                | 6                 |
| K3[Fe(CN)6] (0.45 mM) | 76 | 18               |
| NAD+ (0.1 mM)     | 25                | 0.8               |
| Fumarate (0.05 mM) | 3                | 0.8               |
| NADPH, Q10, Q6, or K2 | 3 | 0.8               |
| None*             | 3                 | 0.8               |

* All reactions contained molecular oxygen at atmospheric pressure, i.e. about 230 μM.
the enzymes purified from *Plasmodium berghei* (Krungkrai et al., 1991) and rat liver (Forman and Kennedy, 1978), and in a recombinant truncated version of the human enzyme (Copeland et al., 1995).

Hitherto, the ability to use NAD\(^{+}\) as electron acceptor seemed to be a unique property of the dihydroorotate dehydrogenase from *Zymobacterium oroticum*, which was discovered by Lieberman and Kornberg (1953). Because this enzyme was made in large amounts when the bacterium was grown with orotate as the sole carbon source and because subsequently discovered dihydroorotate dehydrogenases were unable to use NAD\(^{+}\), the dihydroorotate dehydrogenase of *Z. oroticum* was generally regarded as being an atypical, catabolic enzyme. To the best of our knowledge, however, it was never shown that this enzyme does not also participate in pyrimidine nucleotide biosynthesis. *Z. oroticum* is a Gram-positive bacterium (now called *Clostridium oroticum*), and it is likely that its dihydroorotate dehydrogenase is homologous to the dihydroorotate dehydrogenases of other Gram-positive bacteria (i.e. formed like the δε-enzyme described herein). The reaction kinetics of dihydroorotate dehydrogenase of *Z. oroticum* were studied intensively 30 years ago, and the enzyme was shown to contain two FAD, two FMN, and 4 g-atoms of iron, bound in acid-labile iron-sulfur clusters, in each native enzyme molecule, \(M_r = 120,000\) (Aleman and Handler, 1967; Miller and Massey, 1965). However, no studies were made on the protein moiety of the enzyme, and the amino acid sequence is unknown.

Currently, we are studying how the FMN redox centers on the PyrDB subunit of the δε-enzyme of *L. lactis* interact with the FAD groups and iron-sulfur clusters on the PyrK subunits. The presence of the different types of redox centers on different subunits may facilitate these studies.

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