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Chorismate Mutase and 3-Deoxy-d-arabino-Heptulosonate 7-Phosphate Synthase of the Methylophotrophic Actinomycete Amycolatopsis methanolica

G. J. W. EUVERINK, G. I. HESSELS, C. FRANKE, AND L. DIJKHUIZEN*

Department of Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute (GBB), 9751 NN Haren, The Netherlands

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Chorismate mutase (CM) and 3-deoxy-d-arabino-heptulosonate 7-phosphate (DAHP) synthase (DS) are key regulatory enzymes in l-Phe and l-Tyr biosynthesis in Amycolatopsis methanolica. At least two CM proteins, CMIA and CMIB, are required for the single chorismate mutase activity in the wild type. Component CMIA (a homodimeric protein with 16-kDa subunits) was purified to homogeneity (2,717-fold) and kinetically characterized. The partially purified CMIB preparation obtained also contained the single DS (DSI) activity detectable in the wild type. The activities of CMIA and CMIB were inhibited by both l-Phe and l-Tyr. DSI activity was inhibited by l-Trp, l-Phe, and l-Tyr. A leaky l-Phe-requiring auxotroph, mutant strain GH141, grown under l-Phe limitation, possessed additional DS (DSII) and CM (CMII) activities. Synthesis of both CMII and DSI was repressed by l-Phe. An ortho-m-fluorophenylalanine-resistant mutant of the wild type (strain oFPHE83) that had lost the sensitivity of DSI and CMII synthesis to l-Phe repression was isolated. DSI was partially purified (a 42-kDa protein); its activity was strongly inhibited by l-Tyr. CMII was purified to homogeneity (93.6-fold) and characterized as a homodimeric protein with 16-kDa subunits, completely insensitive to feedback inhibition by l-Phe and l-Tyr. The activity of CMII was activated by CMIB; the activity of CMII plus CMIB was again inhibited by l-Phe and l-Tyr. A tightly blocked l-Phe- plus l-Tyr-requiring derivative of mutant strain GH141-19, that had lost both CMII and CMII activities was isolated. The above-described properties, and the N-terminal amino acid sequences, showed that CMIA and CMII are one and the same protein.

The aromatic amino acids l-Phe, l-Tyr and l-Trp are synthesized via a common pathway. Erythrose-4-phosphate and phosphoenolpyruvate are condensed into 3-deoxy-d-arabino-heptulosonate 7-phosphate (DAHP) by DAHP synthase (DS; EC 4.1.2.15). DAHP is converted via the shikimate pathway into chorismate, involving six enzyme steps (14). Chorismate mutase (CM; EC 5.4.99.5) synthesizes prephenate from chorismate; this synthesis is an important and committed step in l-Phe and l-Tyr biosynthesis and is widespread in nature (4). Chorismate is also converted into anthranilate (1-Tyr biosynthesis), p-aminobenzoate (folic acid biosynthesis), p-hydroxybenzoate (ubiquinone biosynthesis), or isochorismate (menaquinone and enterobactin biosynthesis) (4).

Aromatic amino acid biosynthesis is generally controlled by feedback inhibition or repression at the level of DS and CM. These proteins are present either as monofunctional or bifunctional (iso)enzymes or as part of multienzyme complexes (25).

Current knowledge on the biochemistry and regulation of the pathways of primary metabolism in actinomycetes (gram-positive soil bacteria) is limited but considered to be important for further rational improvement of strains for overproducing aromatic amino acids and derived compounds (7, 29). Many secondary metabolites synthesized by actinomycetes are derived from the aromatic amino acids themselves or from intermediates in their biosyntheses (13, 34). Examples are the antibiotics rifamycin, vancomycin, and avoparcin, which are produced by the industrial actinomycete strains Amycolatopsis mediterranei, Amycolatopsis orientalis, and Amycolatopsis colo- radensis, respectively (16, 22, 25, 30).

We have initiated studies on glucose, methanol, and aromatic amino acid metabolism in the related methylophotrophic actinomycete Amycolatopsis methanolica (9, 16) and have purified and characterized several enzymes of glucose and quinate metabolism, prephenate dehydratase, and the multiple ar-omatic aminotransferases present in this organism (1, 3, 17, 19). Previously, we have also shown that the single DS enzyme (DSI) detectable in wild-type A. methanolica has the unique property of being feedback inhibited by all three aromatic amino acids (12). In this paper we report a detailed biochemical analysis of the DS and CM enzymes of A. methanolica.

MATERIALS AND METHODS

Microorganisms and cultivation. The A. methanolica wild-type strain (NCIB 11946) (9, 28), the plasmid pMEA300 (41a)-deficient strain WV2 (42), and auxotrophic mutants derived from strain WV2 (this study) were used. The procedures followed for the cultivation in batch cultures, harvest of cells, and measurements of growth have been described previously (11). Glucose (1 M) was heat sterilized, and amino acid supplements were filter sterilized.

Mutant isolation. Mutants blocked in aromatic amino acid biosynthesis were isolated following UV irradiation treatments (17, 18).

Isolation of l-Phe analog-resistant mutants. Mutants of the wild-type strain resistant to the toxic l-Phe analog ortho-fluoro-DL-phenylalanine (oFPhe, 27.3 mM) were isolated on 10 mM glucose mineral agar (1.5% [wt/vol]) plates containing the filter-sterilized analog. Fifteen agar plates were inoculated with approximately 5 x 10⁶ cells each. After 2 weeks the spontaneous oFPhe-resistant colonies that had appeared were purified on homologous media.

Preparation of extracts and enzyme assays. Washed cell suspensions were disrupted in a French pressure cell at 1,000 MPa. Unbroken cells and debris were removed by centrifugation at 40,000 x g for 30 min at 4°C. Following desalting through PD 10 Pharmacia columns, the supernatant, containing 10 to 20 mg of protein ml⁻¹, was used for enzyme assays. 3796

* Corresponding author. Mailing address: Department of Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute, Kerklaan 30, 9751 NN Haren, The Netherlands. Phone: 31.50.632153. Fax: 31.50.632154. Electronic mail address: LDIJHUIZEN@BIOL.RUG.NL.
CM was assayed by measuring the amount of prephenate formed after conversion to phenylpyruvate (15). The reaction mixture (100 μl) contained 50 mM Tris-HCl (pH 7.5) and 2.0 mM chorismate and extract or protein, as indicated in the individual experiments. After 10 min, 10 μl of 4.5 M HCl was added, and the reaction mixture was incubated for 15 min at 37°C. The phenylpyruvate formed was extracted by adding 890 μl of a 1.58 M NaOH solution and measuring the $A_{425}$ (phenylpyruvate) = 17.5 × 10$^{-3}$ M$^{-1}$ cm$^{-1}$. Endpoint measurements were indicative of initial reaction rates.

DS was assayed at 37°C by measuring the amount of DAHP formed from 4-phenyl-4-phosphopantoic acid (12, 27).

**Purification of CM from strain W2**. All chromatographic steps were carried out with a System Prep liquid chromatography system (Pharmacia LKB Biotechnology Inc.).

(i) **Step 1.** Glucose-grown cells (25 g [wet weight]) were harvested in the late exponential phase of growth. Extracts were prepared as described above in 25 mM Tris-HCl (pH 7.5) containing 1 mM dithiothreitol (buffer A). DNase I and an extract was prepared in buffer A as described above. DNase I (grade II, from bovine pancreas) and 1 mM MgCl$_2$ were added to the extract, and the mixture was incubated for 10 min.

(ii) **Step 2.** Hydrophobic interaction chromatography. The extract was adjusted to 1.1 M (NH$_4$)$_2$SO$_4$. Precipitated proteins were removed via centrifugation (15 min at 40,000 × g). The resulting supernatant was applied to a column of butyl-Sepharose fast-flow (1.6 by 20 cm, 4°C) equilibrated in buffer A containing 1.1 M (NH$_4$)$_2$SO$_4$. Bound protein was eluted with a 400-ml decreasing linear gradient from 1.1 to 0.3 M (NH$_4$)$_2$SO$_4$ (flow rate, 4.0 ml·min$^{-1}$; fractions, 4.0 ml).

(iii) **Step 3.** Gel filtration chromatography. The protein from step 2 was concentrated by slowly adding solid (NH$_4$)$_2$SO$_4$ to 50% saturation. The mixture was stirred for 30 min at 4°C and centrifuged for 15 min at 40,000 × g. The pellet was dissolved in 3 ml of buffer A and applied to a Superdex 200 (26/60) gel filtration column previously equilibrated in buffer A containing 0.5 M KCl (flow rate, 1 ml·min$^{-1}$; fractions, 2 ml).

(iv) **Step 4.** Anion-exchange chromatography. The protein from step 3 was dialyzed against 50 mM Tris-HCl (pH 8.0) containing 1 mM dithiothreitol (buffer B). Bound protein was eluted with a 30-ml linear increasing gradient from 0 to 0.5 M KCl in buffer B (flow rate, 1 ml·min$^{-1}$; fractions, 0.5 ml).

(v) **Step 5.** Hydrophobic interaction chromatography. The protein from step 3 was dialyzed against 50 mM Tris-HCl (pH 8.0) containing 1 mM dithiothreitol (buffer B) and was applied to a Mono Q (HR 5/5) anion-exchange column. Bound protein was eluted with a 30-ml linear decreasing gradient from 0.5 to 0 M (NH$_4$)$_2$SO$_4$ in buffer B (flow rate, 1 ml·min$^{-1}$; fractions, 0.5 ml).

(vi) **Step 6.** Gel filtration chromatography. The protein from step 2 was applied to a Superdex 200 (26/60) gel filtration column previously equilibrated in buffer B containing 0.15 M KCl (flow rate, 1 ml·min$^{-1}$; fractions, 2 ml).

**Biochemicals.** Chorismate was obtained as a barium salt from Sigma. Before use, barium ions were removed via precipitation with excess K$_2$SO$_4$. Chorismate was further purified on a Ssupelcosil LC-18-DB semi-prep (5-μm particle diameter) column (20.0 cm by 10.0 mm [inside diameter]; Supelco, Bellefonte, Pa.) (8). All other chemicals were analytical grade and commercially available.

## RESULTS

**CM and DS in A. methanolica.** CM and DS activities in extracts of wild-type A. methanolica W2 were 7 and 22 μU·mg$^{-1}$ of protein$^{-1}$, respectively. CM was inhibited by both 1-mM L-Phe and L-Tyr, and DS was inhibited by all three aromatic amino acids (Table 1). The addition of L-Phe, L-Tyr, or L-Trp (100 mg·l$^{-1}$ each), separately or in various combinations, to the growth medium of A. methanolica W2 had no effect on the specific activities of CM and DS and feedback inhibition patterns.

**Purification of CM from wild-type strain W2.** Purification of CM from A. methanolica W2 turned out to be rather difficult. In a first attempt, extract was applied to a Q-Sepharose anion-exchange column. Bound protein was eluted with a linear gradient of 0 to 1 M KCl, but no activity was found. Hydrophobic interaction chromatography of butyl-Sepharose, however, yielded a single activity peak. Also, gel filtration of extracts revealed a single CM activity peak, coeluting with a small DS activity peak, corresponding to a molecular mass of 240 kDa (Fig. 1A). In addition, dialysis against 50 mM Tris-HCl (pH 7.5 or pH 8.8) with or without 1 mM EDTA, centrifugation steps (40,000 to 100,000 × g, 2 h), and ammonium sulfate precipitation did not inactivate CM. Small diffusible effector molecules, e.g., metal ions or metabolites, thus are not required for CM activity. We subsequently observed that after Q-Sepharose anion-exchange chromatography, CM activity could be restored by mixing the flowthrough (component CMa) with fractions eluting from the column at approximately 0.3 M KCl (component CMb). DS activity coeluted with component CMb. Gel filtration of CMa and CMb separately, followed by reconstitution of CM activity with the effector compound, showed that they eluted in fractions corresponding to molecular masses of 31 and 160 kDa, respectively. In this step DS also coeluted with component CMb (160 kDa), instead of behaving as a protein with a size of 240 kDa (see above). Gel filtration of extracts (in the presence of up to 1 M

### Table 1. Specific activities of DS and CM in extracts of glucose-grown cells of A. methanolica W2 and mutants harvested in late exponential phase of growth

<table>
<thead>
<tr>
<th>Strain</th>
<th>CM (μU·mg$^{-1}$)</th>
<th>DS (μU·mg$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type W2</td>
<td>7</td>
<td>22</td>
</tr>
<tr>
<td>GH141</td>
<td>43</td>
<td>50</td>
</tr>
<tr>
<td>GH141+c</td>
<td>7</td>
<td>25</td>
</tr>
<tr>
<td>GH141-19g</td>
<td>0</td>
<td>22</td>
</tr>
<tr>
<td>oFPHE83</td>
<td>33</td>
<td>188</td>
</tr>
<tr>
<td>oFPHE83+c</td>
<td>32</td>
<td>246</td>
</tr>
</tbody>
</table>

$^a$ CM activity was determined without effectors and in the presence of 1 mM L-Phe plus 1 mM L-Tyr. DS activity was determined without effectors and in the presence of 1 mM L-Tyr.

$^b$ CM is also inhibited by 1 mM L-Phe (59%) and 1 mM L-Tyr (50%) separately. DAHP synthase is inhibited not only by L-Tyr (90% at 1.0 mM) but also by L-Phe (74% at 1.0 mM) and L-Tyr (60% at 1.0 mM) separately.

$^c$ Glucose-grown cells supplemented with 100 mg of L-Phe liter$^{-1}$

$^d$ Glucose-grown cells supplemented with 100 μg of L-Tyr plus 100 μg of L-Tyr liter$^{-1}$.

**Analytical methods.** Protein concentrations were determined with the Bio-Rad protein determination kit, with bovine serum albumin as the standard (5). Glucose concentrations were determined with the GOD-peroxidase kit from Boehringer. Amino acid concentrations were determined by HPLC analysis (19).
KCl at pH 7.5) and hydrophobic interaction chromatography did not affect the interaction between CMIa and CMIb, showing that their binding is relatively strong. Gel filtration of extracts at pH 8.5 and 0.15 M KCl did result in separation of the CMIa and CMIb components.

Component CMIa, a minor protein in A. methanolica, was purified (2,717-fold) to homogeneity in four steps, with an overall yield of 5.2% (Table 2). The purification protocol was designed in such a way that CM became separated into components CMIa and CMIb in the last step in the purification scheme only, eluting at 0.05 and 0.35 M KCl, respectively. In this way CM activity could be monitored more conveniently, and homogeneous preparations of CMIa were obtained more easily. CM and DS coeluted during purification; in the final step, DS coeluted with CMIb. The CMIb preparation was not subjected to further purification. SDS-PAGE of CMIa revealed a single band migrating at 16 kDa. In view of its native molecular mass of 31 kDa, CMIa appears to be a homodimeric protein with 16-kDa subunits.

The identification of N-terminal amino acids of CMIa was hampered by the low signal-to-noise ratio. The following seven N-terminal amino acids were tentatively identified: X, X, Q, X, N, E, K, L, T, and P (X, not identified). A BLASTP (2) search of the available databases revealed no significant homology with any other proteins described.

Kinetic studies were carried out with 0.3 μg of protein of pure component CMIa and 20 μg of the CMIb preparation (Fig. 2; Table 2). Under these conditions, CMIa is clearly limiting for the overall CM activity (Fig. 2 inset). The $K_m$ for chorismate was $2.0 \pm 0.3$ mM (mean ± standard deviation). The calculated $V_{max}$ value was $47 \pm 2.9$ U·mg of protein$^{-1}$. CM activity was competitively inhibited by L-Phe and/or L-Tyr. The $K_i$ values for L-Phe and L-Tyr were $0.66 \pm 0.02$ mM and $0.52 \pm 0.01$ mM, respectively.

**Characterization of L-Phe auxotrophic mutants.** A leaky L-Phe auxotrophic mutant, strain GH141, which had lost 90% of the L-Phe aminotransferase activity was isolated previously (1). Cultivation of the strain in glucose mineral medium showed that the doubling time of strain GH141 had increased to 6 h, because growth had become limited by the rate of L-Phe biosynthesis. The doubling time of mutant strain GH141 in glucose mineral medium supplemented with L-Phe (100 mg·liter$^{-1}$) was comparable to that of the wild type (2.5 h). Extracts of mutant strain GH141 cells grown in glucose mineral medium displayed DS and CM activities at levels about 2.5 to 6 times higher than those of the wild type. In mutant strain GH141, CM activity was not feedback inhibited by either L-Phe or L-Tyr, and the sensitivity of DS to L-Trp was strongly reduced (Table 1). Fractionation of extracts of mutant strain GH141, grown on glucose mineral medium, on a Superdex 200 gel filtration column revealed additional CM (CMII, 31 kDa) and DS (DSII, 42 kDa) activity peaks (Fig. 1B). The CMI and DSII enzymes in the wild type and in mutant strain GH141 possessed identical molecular masses and feedback inhibition sensitivities. Cells of strain GH141 grown in glucose mineral medium supplemented with 100 mg of L-Phe liter$^{-1}$ displayed wild-type levels of CM and DS activities and properties (Table 1) and completely lacked the CMI and DSII activity peaks. Strain GH141-19, a tightly blocked L-Phe- plus L-Tyr-requiring auxotrophic mutant, subsequently was derived from strain GH141. Strain GH141-19 had completely lost CM activity (Table 1). The addition of pure component CMIa to extracts of mutant GH141-19 restored CM activity to wild-type levels. This indicates that strain GH141-19 is deficient in component CMIa specifically.

**Characterization of DSII.** Further attempts to purify DSII after gel filtration chromatography failed because of a significant loss of activity, which occurred during anion-exchange or hydrophobic interaction chromatography. The addition of divalent cations to buffer solutions and mixing of the fractions

### Table 2. Purification of CMI from glucose-grown cells of A. methanolica WV2

<table>
<thead>
<tr>
<th>Step</th>
<th>Vol (ml)</th>
<th>Protein (mg)</th>
<th>Total activity (U$^a$)</th>
<th>Sp act (U·mg$^{-1}$)</th>
<th>Purification (fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extract</td>
<td>44</td>
<td>1,562</td>
<td>8.3</td>
<td>0.005</td>
<td>1.0</td>
<td>100.0</td>
</tr>
<tr>
<td>Butyl-Sepharose</td>
<td>38</td>
<td>47.9</td>
<td>2.0</td>
<td>0.042</td>
<td>7.9</td>
<td>24.2</td>
</tr>
<tr>
<td>Superdex 200</td>
<td>8</td>
<td>13.0</td>
<td>1.2</td>
<td>0.091</td>
<td>17.0</td>
<td>14.2</td>
</tr>
<tr>
<td>Mono Q$^b$</td>
<td>1</td>
<td>0.03</td>
<td>0.4</td>
<td>14</td>
<td>2,717.0</td>
<td>5.2</td>
</tr>
</tbody>
</table>

$^a$ One unit of activity is defined as 1 μmol of prephenate formed per min from chorismate.

$^b$ Excess CMIb was added to assay the chorismate mutase activity of CMIa (see the test).
were not successful. Some characteristics of DSII therefore were determined after gel filtration. DSII remained active for at least 24 h at 4°C. The apparent $K_m$ values for phosphoenolpyruvate (3.8 mM erythrose-4-phosphate) and erythrose-4-phosphate (3.0 mM phosphoenolpyruvate) were 0.40 ± 0.06 mM and 1.1 ± 0.2 mM, respectively. With 3.8 mM phosphoenolpyruvate and 1.7 mM erythrose-4-phosphate, DSII activity was inhibited by 1.0 mM l-Trp (17%) and 1.0 mM l-Tyr (83%) but not by l-Phe. l-Tyr inhibition was competitive with respect to the erythrose-4-phosphate concentration, and the $K_i$ value for l-Tyr was 0.031 ± 0.006 mM. Noncompetitive inhibition by l-Tyr was found with respect to the phosphoenolpyruvate concentration, and the $K_i$ value was 0.8 ± 0.2 mM. Intermediates in the biosynthesis of aromatic amino acids (shikimate, anthranilate, chorismate, and prephenate; 1 mM each) inhibited DSII activity by less than 15%.

Characterization of CMII. The CMII enzyme was much more abundant than CMIa in *A. methanolica*. CMII was purified (93.6-fold) to homogeneity from cells of mutant strain GH141, with an overall yield of 25% (Table 3). CMII bound to a Mono Q anion-exchange column at pH 8.8, but not at pH 7.5, and eluted at approximately 0.05 M KCl. SDS-PAGE of CMII revealed a single band migrating at 16 kDa. In view of its native molecular mass of 31 kDa, CMII appears to be a homodimeric protein with 16-kDa subunits.

The following 24 N-terminal amino acids were identified: M, A, Q, T, N, E, K, A, T, P, X, E, T, S, G, E, P, V, A, S, A, X, E, and I (X, not identified). A BLASTP (2) search against the available databases revealed no homology with other CM enzymes described. Six of seven amino acids, however, were identical to tentatively identified N-terminal amino acids of component CMIa (see above).

A virtually linear relationship was observed between the chorismate concentration, in the range of 0 to 4 mM, and CMII activity (Fig. 3). l-Phe and l-Tyr (1 mM concentrations) did not inhibit CMII activity or modify this linear relationship (data not shown). CMII was activated by CMIIb, resulting in Michaelis-Menten kinetics (Fig. 3 inset). The $K_m$ value for chorismate was 2.2 ± 0.2 mM, and a $V_{max}$ of 47 ± 2.5 U · mg of protein $^{-1}$ could be calculated. The activity of CMII plus
CMib was inhibited by l-Phe and l-Tyr (Fig. 3), albeit less strongly than that of CMia plus CMib (Fig. 2). CMII plus CMib activity was competitively inhibited by l-Phe and/or l-Tyr. The \( K_i \) values for l-Phe and l-Tyr were 4.5 ± 0.4 mM and 3.8 ± 0.5 mM, respectively.

**Isolation and characterization of oFPHE83.** De Boer et al. (10) provided evidence that the toxic l-Phe analog oFPhe blocks growth of *A. methanolica* in glucose mineral medium via prephenate dehydratase or CM inhibition. In the present study we observed that oFPhe-resistant mutant strains of wild-type *A. methanolica* (NCIB 11946) could be isolated readily. After 2 weeks of incubation, numerous spontaneous oFPhe-resistant colonies were clearly visible against a background of tiny colonies. Initially, a total of 400 colonies resistant to oFPhe were selected. After repeated transfers, 122 oFPhe-resistant mutants still scored clearly positive. The CM enzymes of five

**TABLE 3. Purification of CMII from glucose-grown cells of *A. methanolica* mutant strain GH141**

<table>
<thead>
<tr>
<th>Step</th>
<th>Vol (ml)</th>
<th>Protein (mg)</th>
<th>Total activity (U)</th>
<th>Sp act (U mg(^{-1}))</th>
<th>Purification (fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extract</td>
<td>49</td>
<td>882.0</td>
<td>47.5</td>
<td>0.05</td>
<td>1.0</td>
<td>100.0</td>
</tr>
<tr>
<td>35–50% (NH(_4))(_2)SO(_4)</td>
<td>8</td>
<td>2.5</td>
<td>50.3</td>
<td>0.13</td>
<td>2.5</td>
<td>105.9</td>
</tr>
<tr>
<td>Superdex 200</td>
<td>24</td>
<td>4.6</td>
<td>21.6</td>
<td>0.25</td>
<td>4.6</td>
<td>45.5</td>
</tr>
<tr>
<td>Mono Q</td>
<td>4</td>
<td>69.5</td>
<td>17.7</td>
<td>3.75</td>
<td>69.5</td>
<td>37.2</td>
</tr>
<tr>
<td>Phenyl-Superose</td>
<td>2</td>
<td>2.3</td>
<td>11.7</td>
<td>5.05</td>
<td>93.6</td>
<td>24.6</td>
</tr>
</tbody>
</table>

* One unit of activity is defined as 1 \( \mu \)mol of prephenate formed per min from chorismate.

![Graph](image-url)  
**FIG. 3.** Specific CM activity of CMII (1.2 \( \mu \)g of protein) (▲) and CMII (0.25 \( \mu \)g of protein) plus excess of component CMib (20 \( \mu \)g of protein) with increasing chorismate concentrations in the absence of an effector (●) or in the presence of 2.0 mM l-Phe (■), 2.0 mM l-Tyr (□), and 2.0 mM l-Phe plus 2.0 mM L-Tyr (▲). (Inset) CM activity with increasing concentrations of CMII in the presence of 20 \( \mu \)g of protein of preparation CMib at a chorismate concentration of 2.0 mM.
oFPhe-resistant strains with normal growth rates in glucose mineral medium were analyzed for their sensitivities towards feedback regulation. In four strains, CM and DS displayed wild-type levels of activities and degrees of sensitivity for feedback regulation. Mutant strain oFPHE83 possessed (very) high levels of CM and DS activities (Table 1). Fractionation of extracts of mutant strain oFPHE83 via gel filtration revealed the additional presence of DSII and CMII enzymes (data not shown). Unlike the situation in the wild type and in mutant strain GH141, the synthesis of the DSII and CMII enzymes was not repressed by L-Phe in mutant strain oFPHE83 (Table 1). The CMIIa and CMIIb enzymes and the DSII and DSII enzymes in the wild-type mutant strain GH141 and in strain oFPHE83 possessed otherwise identical molecular masses and feedback inhibition sensitivities.

**DISCUSSION**

The limited studies thus far carried out with gram-positive bacteria have revealed the presence of isoenzymes of CM in bacilli only (21, 32, 36). In *Bacillus subtilis* one of these CM isoenzymes constitutes a bifunctional protein with DS (35). A CM-DS enzyme complex has also been reported for *Brevibacterium flavum* (37, 40, 41). This paper is the first report of a complex of DS and CM in an actinomycete. CM activity in wild-type *A. methanolica* requires at least two proteins (CMIIa and CMIIb), and in several purification steps coelution of DS (DSII) and CM occurred (this study). In the final step of the purification protocol for component CMIIa, DSII coeluted with CMIIb. Also, the molecular masses of DSII (168 kDa [12]) and CMIIb (160 kDa) are similar. These data thus suggest that CMIIb and DSII are identical. Considering the molecular masses of components CMIIa and CMIIb-DSII, two CMIIa dimers (31 kDa) are most likely associated with CMIIb-DSII in a 240-kDa complex (Fig. 1A).

CM activity in *A. methanolica* is feedback inhibited by L-Phe and L-Tyr, a characteristic that is shared with the enzyme from *A. mediterranei* (43). The *Corynebacterium glutamicum* (24) and *B. flavum* (40) CM enzymes were also inhibited by L-Phe and L-Tyr; this inhibition was released in the presence of L-Trp, a characteristic not shown by *A. methanolica* CM. The spore-forming members of the order Actinomycetales display CM activity that is inhibited by L-Tyr and/or L-Trp only (26, 38). The CM of *Streptomyces aureofaciens* contains at least three subunits with sizes of 14 kDa; the enzyme is insensitive to inhibition by aromatic amino acids (20). Also, the 75-kDa CM detected in extracts of *Streptomyces* sp. strain 3022a, a chloramphenicol producer, was not inhibited by aromatic amino acids (33).

Characterization of the leaky L-Phe aminotransferase mutant strain GH141 (1) revealed that synthesis of a second CM enzyme (CMII) and a second DS enzyme (DSII) became de-
repressed under L-Phe-limiting conditions. Following growth in the presence of L-Phe, only the constitutive DSII with L-Trp and L-Tyr activation of prephenate dehydratase activity, (ii) L-Phe and L-Tyr inhibition of CM activity, and (iii) repression of CMII-CMIa and DSII synthesis by L-Phe (Fig. 4). Under L-Phe-limiting conditions, derepression of both CMII-CMIa and DSII synthesis increases the flux of intermediates toward L-Phe and L-Tyr biosynthesis. L-Tyr inhibition of DSII will prevent the accumulation of L-Tyr under L-Phe limitation (Fig. 4). The relative in vivo contribution of these control mechanisms remains to be determined.

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REFERENCES