Isocitrate lyase from *Phycomyces blakesleeanus*

The role of Mg$^{2+}$ ions, kinetics and evidence for two classes of modifiable thiol groups

Javier RÚA, Dolores de ARRIAGA, Félix BUSTO and Joaquin SOLER*
Departamento de Bioquímica y Biología Molecular, Universidad de León, 24007 León, Spain

Isocitrate lyase was purified from *Phycomyces blakesleeanus* N.R.R.L. 1555(—). The native enzyme has an *M*$_{r}$ of 240000. The enzyme appeared to be a tetramer with apparently identical subunits of *M*$_{r}$ 62000. The enzyme requires Mg$^{2+}$ for activity, and the data suggest that the Mg$^{2+}$-isocitrate complex is the true substrate and that Mg$^{2+}$ ions act as a non-essential activator. The kinetic mechanism of the enzyme was investigated by using product and dead-end inhibitors of the cleavage and condensation reactions. The data indicated an ordered Uni Bi mechanism and the kinetic constants of the model were calculated. The spectrophotometric titration of thiol groups in *Phycomyces* isocitrate lyase with 5,5'-dithiobis-(2-nitrobenzoic acid) gave two free thiol groups per subunit of enzyme in the native state and three in the denatured state. The isocitrate lyase was completely inactivated by iodoacetate, with non-linear kinetics. The inactivation data suggest that the enzyme has two classes of modifiable thiol groups. The results are also in accord with the formation of a non-covalent enzyme–inhibitor complex before irreversible modification of the enzyme. Both the equilibrium constants for formation of the complex and the first-order rate constants for the irreversible modification step were determined. The partial protective effect of isocitrate and Mg$^{2+}$ against iodoacetate inactivation was investigated in a preliminary form.

**INTRODUCTION**

Isocitrate lyase (EC 4.1.3.1) catalyses the reversible aldol cleavage of *threo-D, (+)-*isocitrate to succinate and glyoxylate. It is a key enzyme of the glyoxylate pathway, which is used by micro-organisms growing on C$_{4}$ compounds to generate the precursors necessary for biosynthesis. The enzyme in *Escherichia coli* and other enteric bacteria plays an important role in the regulation of isocitrate flux at the branch-point between the glyoxylate pathway and the tricarboxylic acid cycle [1–5]. Isocitrate lyases have been purified from a restricted number of species, among which are *E. coli* [6], *Pseudomonas indigofera* [7], *Chlorella pyrenoidosa* [8], *Neurospora crassa* [9] and a thermophilic *Bacillus* [10]. Although several physical and kinetic properties of the enzymes from these sources have been characterized, the true substrate still remains to be established. There are few reports about the role of Mg$^{2+}$ in isocitrate lyase kinetics. Giachetti et al. [11] have found that the Mg$^{2+}$-isocitrate complex is the true substrate for the *Pinus pinea* isocitrate lyase, in contrast with the commonly accepted substrate D$_{L}$-isocitrate. Little is known about the structure and active site of tetrameric isocitrate lyase. The presence of a cysteine residue has been reported in the enzymes from *Ps. indigofera* [12], castor bean (*Ricinus communis*) [13] and *E. coli* [14].

So far little has been learnt about the regulation of isocitrate flux in filamentous fungi [15,16], and no details of the purification of *Phycomyces blakesleeanus* isocitrate lyase have hitherto been reported, nor have its kinetic and regulatory properties been examined.

In the present paper we report the purification and the physicochemical properties of isocitrate lyase from *Ph. blakesleeanus* N.R.R.L. 1555(—). We also present a preliminary study of the effect of Mg$^{2+}$ ions on the enzyme and a detailed analysis of its kinetic mechanism. Finally we present a study of the thiol groups, with an analysis of protection against inactivation, which allows us to discuss the role of these thiol groups.

**EXPERIMENTAL**

**Materials**

*threo-DL*-Isocitrate (trisodium salt), *threo-D*-isocitrate (monopotassium salt), succinic acid, glyoxylic acid, phenylhydrazine hydrochloride, 6-phosphogluconate (tricyclohexylammonium salt), phosphonopyruvate (monocyclohexylammonium salt), oxalic acid (sodium salt), SDS, Mops, iodoacetate, DEAE-cellulose and Blue Dextran were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Sephadex G-200, *M*$_{r}$ markers for analytical gel filtration and *M*$_{r}$ markers for SDS/PAGE were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Reagents for gel electrophoresis were from Shandon Southern Products, Cheshire, U.K. Pig heart isocitrate dehydrogenase (EC 1.1.1.42), NADP$^{+}$ (disodium salt), dithiothreitol and 5,5'-dithiobis-(2-nitrobenzoic acid) were from Boehringer Mannheim, Mannheim, Germany. Fumaric acid was obtained from Fersona, Barcelona, Spain. All other chemicals used were standard analytical grade and provided by Merck, Darmstadt, Germany.

**Isocitrate lyase assays**

The cleavage reaction catalysed by isocitrate lyase was assayed by a modification of the continuous method described by Dixon & Kornberg [17]. The standard reaction mixture comprised 25 mM-imidazole/HCl buffer, pH 6.8, containing 5 mM-MgCl$_{2}$, 4 mM-phenylhydrazine hydrochloride, 4 mM-*threo-DL*-isocitrate and enzyme, in a final volume of 1 ml. The reaction was started by the addition of isocitrate, and the isocitrate cleavage was measured at 30 °C by the change in absorbance at 324 nm associated with the formation of glyoxylate phenylhydrazone (ε = 14.63 × 10$^{7}$ M$^{-1}$·cm$^{-1}$). A unit of enzyme activity is defined as the amount of enzyme that catalyses the formation of 1 μmol of glyoxylate phenylhydrazone/min, under standard conditions.

The condensation reaction catalysed by isocitrate lyase was assayed by coupling the formation of isocitrate to the reduction of NADP$^{+}$ by using isocitrate dehydrogenase. The standard
reaction mixture comprised 25 mM-Mops/NaOH buffer, pH 7.5, containing 5 mM-MgCl₂, 2 mM-NADP⁺, isocitrate dehydrogenase (0.4 unit/ml), succinate, glyoxylate as indicated and enzyme, in a final volume of 1 ml. The reaction was started by the addition of purified isocitrate lyase, and NADPH formation was monitored at 30 °C by the change in absorbance at 340 nm (ε = 3.26 × 10³ M⁻¹ cm⁻¹, which was determined experimentally for our assay conditions). A unit of enzyme activity is defined as the amount of enzyme that catalyses the formation of 1 μmol of NADPH/min, under standard conditions.

Protein concentration was measured by the method of Lowry et al. [18] or by the method of Warburg & Christian described by Layne [19]; BSA was used as standard.

In all kinetic experiments we used three-ν-s-isocitrate. All initial-rate data were plotted in double-reciprocal form and were fitted to the Michaelis–Menten equation by using the Enzfit computer program [20], after graphically checking that the double-reciprocal plots were linear. Slopes and intercepts in secondary replots were fitted to straight lines by linear regression by using the above computer program. Parameters and the corresponding standard errors were calculated by the program. Initial-rate data were also fitted to eqn. (1):

\[
v = \frac{V_p^{max} [A] - [P][Q]}{K_{eq}^{max} + [A] + V_p^{max} K_{eq}^{max} + \frac{V_p^{max} K_{eq}^{max}}{K_{eq}^{max}} + \frac{V_p^{max} K_{eq}^{max}}{K_{eq}^{max}} + \frac{V_p^{max} K_{eq}^{max}}{K_{eq}^{max}} + \frac{V_p^{max} K_{eq}^{max}}{K_{eq}^{max}}}
\]

which described the complete velocity equation for an ordered Uni Bi mechanism (Scheme 1) according to Segel [21], where A represents isocitrate, P represents succinate, Q represents glyoxylate and \( V_p^{max} \) and \( V_p^{max} \) are the maximum velocities for cleavage reaction and condensation reaction respectively.

Thiol group determination

The purified isocitrate lyase was treated in the native state, or after denaturation with 1% (w/v) SDS or 8 M-urea, with 10 mM-5,5'-dithiobis-(2-nitrobenzoic acid) and 1 mM-EDTA in a total volume of 1 ml, and the maximum absorbance at 412 nm attained (after about 10 min) was recorded against a suitable reagent blank. From the slopes of the plots of \( A_{\text{abs}} \) versus protein concentration and assuming a molar absorption coefficient of 13.6 × 10³ M⁻¹ cm⁻¹ [22] for 5-mercapto-2-nitrobenzoate, the number of thiol groups was calculated.

Reaction of isocitrate lyase with iodoacetate

Solutions of iodoacetate were prepared before each experiment by dissolving the solid in water and neutralizing it with NaOH. Purified Phycomyces isocitrate lyase was desalted through Sephadex G-25, a PD-10 disposable column equilibrated with 50 mM-imidazole/HCl buffer, pH 6.8, containing 1 mM-EDTA. The reaction of isocitrate lyase with iodoacetate was carried out at 25 °C in the above-cited buffer. The inactivation was monitored by periodic activity assay of samples of the incubation mixture as described above. The concentration of iodoacetate present during the activity assay (≤ 0.05 mM) had no effect on enzyme activity.

Data for protection against inactivation were analysed by plots of \( \Delta k \) versus [ligand] and of \( 1/\Delta k \) versus 1/[ligand], where \( \Delta k \) is the difference between \( k_{\text{app}} \), in the absence of the protecting ligand and \( k_{\text{app}} \), in the presence of the protecting ligand, and \( k_{\text{app}} \) is the inactivation rate constant. \( \Delta k_{\text{max(app.)}} \) was obtained by extrapolating the plot of 1/\( \Delta k \) versus 1/[ligand] to the ordinate axis. The limiting inactivation constant representing the \( k_{\text{app}} \) at the saturating concentration of protecting ligand can be calculated from \( k_{\text{app}} \), in the absence of the protecting ligand and \( \Delta k_{\text{max(app.)}} \). The dissociation constant of ligand was obtained by extrapolating the plot of 1/\( \Delta k \) versus 1/[ligand] to the abscissa axis.

Isocitrate lyase purification

Ph. blakesleeanus wild-type strain N.R.R.L. 1555(-) was grown in a liquid minimal medium [23], except that we used 2.73 % acetate as the carbon source and l-asparagine (2 g/l) as nitrogen source. The mycelium was harvested after 24–28 h of growth as described previously [16].

All the purification steps were performed at 0–4 °C. The mycelia of Ph. blakesleeanus were cut into pieces and suspended in buffer A (50 mM-imidazole/HCl buffer, pH 7.5, containing 10 mM-KCl, 1 mM-EDTA, 1 mM-MgSO₄ and 1 mM-dithiothreitol) at a ratio of 5 ml/g wet wt. and homogenized in a Braun MSK homogenizer cell-disruptor for 35 s. The homogenate was centrifuged at 104000 g for 60 min. The supernatant was adjusted to 35 % saturation with (NH₄)₂SO₄ by slowly adding solid (NH₄)₂SO₄. The material was stirred for 20 min and centrifuged at 15000 g for 20 min, and the pellet was discarded. Further solid (NH₄)₂SO₄ was added to the supernatant to give 60 % saturation, and the preparation was stirred and centrifuged as above. The pellet was dissolved in a small volume of buffer B (20 mM-imidazole/HCl buffer, pH 7.0, containing 5 mM-KCl, 1 mM-MgSO₄, 1 mM-EDTA and 1 mM-dithiothreitol). The suspension was loaded on to a column (3.5 cm × 50 cm) of Sephadex G-200 equilibrated with buffer B, at a flow rate of 20 ml/h. Fractions (2.5 ml) containing isocitrate lyase activity were pooled and loaded on to a column (2 cm × 23 cm) of DEAE-cellulose equilibrated with buffer B, and fractions (1 ml) containing isocitrate lyase activity were eluted with the same buffer at a flow rate of 20 ml/h; the remaining protein fractions (without enzymic activity) were eluted from the column by increasing the molarity of buffer B by adding 0.5 M-KCl. The active fractions were pooled in buffer B containing BSA (0.5 mg/ml) and 5 mM-dithiothreitol, and stored at 4 °C.

Analytical gel filtration

A column (2.7 cm × 40 cm) of Sephadex G-200 was equilibrated with buffer B. The markers used were ferritin (Mr, 440000), catalase (Mr, 232000), fructose-bisphosphate aldolase (Mr, 158000), BSA (Mr, 67000), ovalbumin (Mr, 43000) and chymotrypsinogen A (Mr, 25000).

PAGE

PAGE under non-denaturing conditions was performed on 7.5 % acrylamide rods (pH 8.3) by a technique essentially similar to that of Davis [24]. SDS/PAGE was carried out on 140 cm × 140 cm × 2 cm gel slabs (10 % acrylamide and 0.1 % SDS) in phosphate/SDS buffer according to the Weber & Osborn method [25]. The subunit Mr markers used were α-lactalbumin (Mr, 144000), trypsin inhibitor (Mr, 20100), carbonic anhydrase.

\[
\begin{align*}
\text{E} + \text{A} & \overset{k_{1s}}{\underset{k_{-1}}{\rightleftharpoons}} (\text{EA} \overset{k_{2s}}{\underset{k_{-2}}{\rightleftharpoons}} \text{EPQ}) \overset{k_{3s}}{\underset{k_{-3}}{\rightleftharpoons}} \text{P} + \text{EQ} \overset{k_{4s}}{\underset{k_{-4}}{\rightleftharpoons}} \text{E} + \text{Q}
\end{align*}
\]

Scheme 1.
Table 1. Purification procedure for isocitrate lyase from the mycelium of *Ph. blakesleeanus* N.R.R.L. 1555(−)

The homogenate was obtained from 20 g of myelia. For experimental details see the text.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein (mg)</th>
<th>Total activity (units)</th>
<th>Specific activity (units/mg)</th>
<th>Purification factor (fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>3540</td>
<td>177.0</td>
<td>0.05</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>104000 g supernatant</td>
<td>573</td>
<td>143.3</td>
<td>0.25</td>
<td>5.0</td>
<td>81</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ fractionation</td>
<td>495</td>
<td>139.0</td>
<td>0.28</td>
<td>5.6</td>
<td>79</td>
</tr>
<tr>
<td>0-35% saturation</td>
<td>299</td>
<td>98.7</td>
<td>0.33</td>
<td>6.6</td>
<td>56</td>
</tr>
<tr>
<td>35-60% saturation</td>
<td>57</td>
<td>61.6</td>
<td>1.08</td>
<td>21.6</td>
<td>35</td>
</tr>
<tr>
<td>DEAE-cellulose eluate</td>
<td>6</td>
<td>56.0</td>
<td>9.34</td>
<td>187</td>
<td>32</td>
</tr>
</tbody>
</table>

(Mᵣ 30000), ovalbumin (*Mᵣ* 43000), BSA (*Mᵣ* 67000) and phosphorylase b (*Mᵣ* 94000).

**RESULTS AND DISCUSSION**

**Purification and physicochemical properties of isocitrate lyase**

The results of a typical purification procedure for isocitrate lyase from mycelium of *Ph. blakesleeanus* are summarized in Table 1. We used the ultracentrifugation step instead of a 20000 g centrifugation one because it produces a higher increase in the specific activity of the enzyme (about 2-fold). The elution profiles of an ion-exchange chromatography on a DEAE-cellulose column are shown in Fig. 1. All isocitrate lyase activity was contained in the minor protein peak eluted with the equilibration buffer (buffer B), whereas a major protein peak was eluted from the column on increasing the buffer molarity by the addition of 0.5 m-KCl. This step was the more effective one in the purification procedure. The use of a low-molarity buffer (5 mM- or 10 mM-Tris/HCl buffer) and different pH (range pH 6.8-7.9) in this chromatography step did not change the elution profile of enzyme. The yield and final purification factor were 30% and 187-fold respectively.

The purified *Phycomyces* isocitrate lyase seems to be homogeneous as judged by the existence of the single protein band with a relative mobility of 0.22 observed on PAGE (7.5% acrylamide) in non-denaturing conditions. A high degree of homogeneity was also indicated by using PAGE in the presence of SDS, as evidenced by the existence of a single band with a relative mobility of 0.23. From the position of this band, the subunit *Mᵣ* was estimated to be 62000±2000 (Fig. 2). Gel filtration on a Sephadex G-200 column with several standard proteins gave an *Mᵣ* for native enzyme of 240000±5000. Thus isocitrate lyase from *Ph. blakesleeanus*, like the enzyme from other sources [26], is a tetramer of identical or similar subunits. The Stokes radius of the enzyme calculated as described by Ackers [27] was 5.4 nm. From this value, we have estimated for the diffusion coefficient (*D*) a value of 3.97 × 10⁻⁹ cm²·s⁻¹. The value of both parameters is close to those described for the enzymes from *Bacillus* sp. [10], *Ps. indigofera* [7] and *C. pyrenoidosa* [8].

The activity of *Phycomyces* isocitrate lyase was very unstable, especially after the DEAE-cellulose step, even at −20 °C and in the presence of glycerol, unlike the enzymes from the thermophilic *Bacillus* sp. [10], *Ps. indigofera* [7] and *E. coli* [6]. This problem was overcome by the addition of 5 mm-dithiothreitol and 0.5 mg of BSA/ml. Under these conditions the enzymic preparation of *Ph. blakesleeanus* isocitrate lyase could be stored at 4 °C for 15

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Fig. 1. DEAE-cellulose chromatography of isocitrate lyase from *Ph. blakesleeanus* N.R.R.L. 1555(−)

The column was run as described in the Experimental section. O, Isocitrate lyase activity; ●, *A₄₉₀*. Fractions 35-55 were pooled. The assays for the isocitrate cleavage were carried out as described in the Experimental section, with 10 µl of each 1 ml fraction of the enzyme preparation.

Fig. 2. SDS/PAGE of isocitrate lyase from *Ph. blakesleeanus* N.R.R.L. 1555(−)

Tracks 1 and 5, *Mᵣ* markers (*Mᵣ* values indicated at the right). Track 3, enzyme after elution from the DEAE-cellulose column.
days without significant loss of activity. The presence of reducing agents was also necessary to preserve the isocitrate lyase activity in enzymes from other sources [26,28]. On the other hand, Mg$^{2+}$ ions protected *Phycomyces* isocitrate lyase from inactivation during storage and heat-inactivation, as is the case with the enzyme from the pollen of *Pinus densiflora* [29]. Although *Phycomyces* isocitrate lyase activity was decreased to about 50% by heating at 40 °C for 5 min, the activity was entirely stable with the same treatment in the presence of 5 mM-MgCl$_2$.

From an Arrhenius plot (Fig. 3) we have obtained two apparent activation energy ($E_a$) values for isocitrate cleavage catalysed by the *Phycomyces* enzyme. Allowance was made for the change with temperature in the pH of the buffer. The apparent activation energy ($E_a$) at lower temperatures (below 40°C) was 51.6 kJ·mol$^{-1}$ which is the same value as that reported for the *Ps. indigofera* isocitrate lyase [30], but lower than that estimated for *Bacillus* sp. enzyme [10]. The apparent activation energy ($E_a$) at the higher temperatures was 9.5 kJ·mol$^{-1}$, 40 °C being the transition temperature. Several explanations as to why the Arrhenius plot is concave downwards have been suggested [31]. A temperature-dependent equilibrium between two active enzymic forms with different activation energies may be a valid explanation for *Phycomyces* isocitrate lyase if we take into account the role played by Mg$^{2+}$ ions, as described below.

The activity of purified isocitrate lyase from *Ph. blakesleeanus* was dependent on ionic strength ($I$) (when NaCl is added). It decreased from 10.01 (maximum activity) to 10.1 (about 5% residual activity) in imidazole/HCl buffer, pH 6.8. Optimum pH for activity of the cleavage reaction was between pH 6.5 and pH 7.0 in 25 mM-imidazole/HCl buffer and 25 mM-sodium phosphate buffer, whereas for the condensation (reverse) reaction the pH optimum was between pH 7.5 and pH 8.0, in good agreement with that described for isocitrate lyases from other sources [26].

The isocitrate lyase from *Phycomyces* was inactivated by urea at 30 °C in 25 mM-sodium phosphate buffer, pH 7.0, containing 1 mM-EDTA for 5 min of incubation. It must be pointed out that a decline of activity from 80% to 3% was obtained as the urea concentration was raised from 0.5 M to 3.0 M. These results are in accordance with those obtained for *Ps. indigofera* enzyme, which showed 96% inactivation with 3 M-urea [32].

The assays were carried out in 25 mM-imidazole/HCl buffer, pH 6.8, containing 5 mM-MgCl$_2$, at 4 mM-$\delta$-isocitrate.

### Effect of Mg$^{2+}$ ions on *Phycomyces* isocitrate lyase

Mg$^{2+}$ was essential for the cleavage of isocitrate catalysed by *Ph. blakesleeanus* isocitrate lyase as has been reported for all other isocitrate lyases studied [26]. When *Phycomyces* isocitrate lyase activity was assayed from a mycelial extract obtained by homogenization of the mycelium in a buffer without Mg$^{2+}$ and further desalted through a Sephadex G-25 PD-10 disposable column (Pharmacia), no activity was observed in the absence of any added Mg$^{2+}$. Mg$^{2+}$ can be partially replaced by Co$^{2+}$ and Mn$^{2+}$, which yielded 40% and 29% respectively of the activity with Mg$^{2+}$. Similar partial activation by Co$^{2+}$ and Mn$^{2+}$ in the absence of Mg$^{2+}$ has been reported for the *Ps. indigofera* enzyme [33] and the flax (*Linum usitatissimum*) enzyme [34].

Mg$^{2+}$ was postulated to be necessary for catalysis on the active site of isocitrate lyase [35], but at higher concentrations Mg$^{2+}$ has an inhibitory effect [26]. From studies carried out with *Ricinus* enzyme, Malhotra *et al.* [36] have proposed the existence of two classes of Mg$^{2+}$-binding sites with different affinities. We have obtained for *Phycomyces* isocitrate lyase the experimental initial-velocity curves as a function of total Mg$^{2+}$ concentration at different fixed total threeo-$\delta$-isocitrate concentrations (Fig. 4), and as a function of total threeo-$\delta$-isocitrate concentration at different fixed total Mg$^{2+}$ concentrations (results not shown here). These results are qualitatively consistent with the theoretical profiles obtained by Giachetti *et al.* [11] with *Pinus pinea* isocitrate lyase, assuming that Mg$^{2+}$-isocitrate complex is the true substrate for the enzyme and that Mg$^{2+}$ ions act as a non-essential activator. The model supposes an activator Mg$^{2+}$-binding site with high affinity in addition to the catalytic site with lower affinity. Furthermore, Mg$^{2+}$ binds at the catalytic site of the activated enzyme (Mg$^{2+}$-enzyme) only, and free isocitrate at the catalytic site of the non-activated enzyme only. Both Mg$^{2+}$-enzyme–Mg$^{2+}$ and enzyme–isocitrate complexes are non-effective. On the other hand, the velocity curve obtained with equimolar total threeo-$\delta$-isocitrate concentrations and total Mg$^{2+}$ concentrations (results not shown here) is sigmoidal. This sigmoidicity was apparent at concentrations (below 0.1 mM) lower than the value obtained for $K_m$ (Mg$^{2+}$-isocitrate complex dissociation constant) of 0.78 mM [11]. Thus increasing total [threeo-$\delta$-isocitrate] and total [Mg$^{2+}$] by a given factor not only increases the concentration of true substrate, Mg$^{2+}$–isocitrate complex, by

![Fig. 4. Experimental initial-velocity profiles for the isocitrate cleavage catalysed by *Ph. blakesleeanus* isocitrate lyase, as a function of total [Mg$^{2+}$].](image-url)

The assays were carried out at 30 °C in 25 mM-imidazole/HCl buffer, pH 6.8, at fixed 0.01 mM (●) and 1 mM (○) total threeo-$\delta$-isocitrate.
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A greater factor, but also increases the concentration of the activator, free Mg$^{2+}$. At 0.05 mM total isocitrate and 0.05 mM total Mg$^{2+}$, the equilibrium concentration of Mg$^{2+}$-isocitrate complex is 2.84 μM and [free Mg$^{2+}$] is 47.2 μM, whereas at 0.1 mM total isocitrate and 0.1 mM total Mg$^{2+}$, the equilibrium concentration of Mg$^{2+}$-isocitrate complex is 10 μM and [free Mg$^{2+}$] is 89.7 μM. In addition, with a concentration range of total [Mg$^{2+}$] between 50 μM and 7 mM with a fixed 1 mM total three-D$_{1}$-isocitrate concentration, we have estimated the concentrations of free Mg$^{2+}$. From a double-reciprocal plot we have estimated an apparent dissociation constant value for free Mg$^{2+}$ of 0.1 mM. With regard to this effect, Segel [37] has emphasized that the activation system in which activator-substrate complex is the true substrate can yield sigmoidal velocity curves, even when there are no multiple interacting sites. Thus, assuming the model proposed by Giachetti et al. [11], we have estimated the apparent $K_m$ for Mg$^{2+}$-isocitrate complex at fixed total [Mg$^{2+}$] of 0.01, 2 and 20 mM and variable 0.01–2 mM total [three-D$_{1}$-isocitrate]. From double-reciprocal plots of velocity data versus [Mg$^{2+}$-isocitrate] we have estimated the apparent $K_m$ values of 1, 39 and 156 μM respectively. At the lower fixed total [Mg$^{2+}$], the enzyme would already be mostly in the enzyme–Mg$^{2+}$-isocitrate complex form, and increasing the fixed total [Mg$^{2+}$] the enzyme would be distributed between the Mg$^{2+}$-enzyme–Mg$^{2+}$-isocitrate, the Mg$^{2+}$-enzyme–Mg$^{2+}$ and the enzyme–Mg$^{2+}$-isocitrate forms. However, the apparent $V_{max}$ value at fixed 2 mM total [Mg$^{2+}$] was 1.6 times that obtained at 0.01 mM total [Mg$^{2+}$]. At fixed 20 mM total [Mg$^{2+}$] the presence of the non-effective Mg$^{2+}$-enzyme–Mg$^{2+}$ complex counteracted the increase in the apparent $V_{max}$ and the value obtained was similar to that obtained at 0.01 mM total [Mg$^{2+}$]. From the kinetic studies shown below, we have calculated a $K_m$ value of Phycomyces isocitrate lyase for three-D$_{1}$-isocitrate of 58 μM in 25 mM-imidazole/HCl buffer, pH 6.8, containing 5 mM-MgCl$_2$ (Table 2). The fact that this value of $K_m$ calculated for three-D$_{1}$-isocitrate is close to the mean values for the Mg$^{2+}$-isocitrate complex indicates that this model may be a good approach to understanding the role of Mg$^{2+}$ ions in the regulation of Phycomyces isocitrate lyase.

**Kinetic mechanism of Phycomyces isocitrate lyase**

Initial-velocity studies were performed for the condensation (reverse) reaction and the cleavage reaction in the absence of reaction products. The condensation reaction was examined at various concentrations of both glyoxylate (0.0125–4 mM) and succinate (0.125–20 mM). Substrate inhibition was observed for glyoxylate concentrations above 0.7 mM and succinate concentrations above 2 mM. Linear double-reciprocal plots were obtained when the concentration of either glyoxylate or succinate (in the substrate concentration range at which substrate inhibition was not detectable) was varied at fixed concentrations of the other substrate. In both cases the extrapolated straight lines intersected in the upper-left quadrant (Fig. 5). Secondary replots of intercepts and slopes were linear with the reciprocal of concentrations of glyoxylate and succinate.

This kinetic pattern indicates that the condensation reaction proceeds by a sequential reaction mechanism, which is qualitatively consistent either with an ordered Bi Uni mechanism or

**Table 2. Kinetic constants for isocitrate lyase from Ph. blakesleeanus**

All kinetic constants were determined by fitting the data to eqn. (1). To compare the values of $V_{max}$ in both directions, they were expressed per minute, taking 218,000 as the $M_r$ of the enzyme.

<table>
<thead>
<tr>
<th>Substrate (pH 7.5)</th>
<th>Michaelis constants</th>
<th>Dissociation constants</th>
<th>$10^{-3} \times V_{max}$ (min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Condensation reaction</td>
<td>$K_m^{max}$</td>
<td>0.65 ± 0.06 mM</td>
<td>$K_m^{min}$</td>
</tr>
<tr>
<td>Succinate</td>
<td>$K_m^{max}$</td>
<td>28 ± 0.2 μM</td>
<td>$K_m^{min}$</td>
</tr>
<tr>
<td>Glyoxylate</td>
<td>$K_m^{max}$</td>
<td>58 ± 1 μM</td>
<td>$K_m^{min}$</td>
</tr>
</tbody>
</table>

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with a rapid-equilibrium random Bi Uni mechanism [21,38]. The kinetic mechanism was probed further by the analysis of inhibition patterns obtained by using dead-end inhibitors, namely phosphoenolpyruvate, itaconate and oxalate. Here we used the Segel nomenclature for inhibition types [39]. Inhibition by isocitrate as the reaction product could not be tested because of interference with the assays. Phosphoenolpyruvate and itaconate have been used as analogues of succinate in studies of other isocitrate lyases [6,30,40-42]; they were linear competitive inhibitors with regard to glyoxylate and linear competitive inhibitors with respect to succinate for Phycomyces isocitrate lyase. Oxalate, an analogue of glyoxylate [42-44], gave a linear competitive inhibition for glyoxylate and a linear mixed-type inhibition for succinate.

These results support an ordered binding mechanism for the condensation reaction for Phycomyces isocitrate lyase in which glyoxylate is bound first to the enzyme, with subsequent binding of succinate. In an ordered Bi Uni mechanism in which glyoxylate adds first and succinate afterwards, a dead-end inhibitor competitive with succinate will be uncompetitive with glyoxylate [45,46].

On the other hand, with respect to the cleavage reaction catalysed by Phycomyces isocitrate lyase, succinate was a linear mixed-type inhibitor (Fig. 6a), as expected in an ordered Uni Bi mechanism [21]. Oxalate was a linear competitive inhibitor with respect to isocitrate (Fig. 6b), whereas phosphoenolpyruvate and itaconate were linear uncompetitive inhibitors. On the basis of these results we think that the kinetic mechanism followed by Phycomyces isocitrate lyase is ordered Uni Bi, and thus is the same as that proposed for the enzymes from N. crassa [9], Ps. indigofera [40], Bacillus sp. [10] and Lupinus [47]. Mackintosh & Nimmo [6] have reported a random-order equilibrium mechanism for the E. coli enzyme. However, since the phosphoenolpyruvate and itaconate inhibition for the cleavage reaction appeared to be non-competitive or mixed-type at higher concentrations, it would seem that the kinetic mechanism could be preferentially ordered like that proposed for the enzymes from water-melon cotyledons [42] and from Linum usitatissimum [48]. Table 2 summarizes the kinetic constants calculated for Phycomyces isocitrate lyase in both directions of reaction, on the basis of an ordered Uni Bi mechanism and from the fit of the data shown in Figs. 5 and 6(a). The $K_n$ value of Ph. blakesleeanae isocitrate lyase for three-$\theta$-isocitrate (58 $\mu$m) was similar to the values reported for the enzymes from N. crassa [9], E. coli [6], C. pyrenoidosa [8] and Bacillus sp. [10] and far lower than the values of 1.2, 0.82, 0.45, 0.29 and 0.25 mm reported for the enzymes from yeast [7], Ps. indigofera [7,33], Pseudomonas aeruginosa [7], Linum usitatissimum [34] and water-melon cotyledons [49]. The $K_n$ values for succinate and glyoxylate were also in agreement with those reported for the enzymes from E. coli [6], N. crassa [9] and Lupinus [28]. $K_i$ data for dead-end inhibitors are summarized in Table 3.

In addition, we have calculated the rate constant values for the individual steps of the reaction shown in Scheme 1, which rule out the isomerization step of the central complex according to Segel [21], these values being as follows: $k_{+1} = (61 \pm 4.6) \times 10^9$ mm$^{-1}$ min$^{-1}$, $k_{-1} = (14 \pm 1.1) \times 10^9$ min$^{-1}$, $k_{+2} = (33 \pm 2) \times 10^9$ min$^{-1}$ and $k_{-2} = (54 \pm 3.9) \times 10^9$ mm$^{-1}$ min$^{-1}$. The $k_{-2}$ value could not be calculated because of the negative value obtained for $k_{-2}$ since the $k_{+2}$ value was close to the $V_{max}$ value. Judged from the estimated values for the individual reaction rate constants, it seems reasonable to suppose that the dissociation of glyoxylate from the enzyme-glyoxylate complex (cleavage reaction) and the dissociation of isocitrate from enzyme-isocitrate complex (condensation reaction) would be the slowest step in each direction of the reaction catalysed by isocitrate lyase from Ph. blakesleeanae.
maleate than by fumarate, although the inhibition pattern shown by maleate was different.

Citrate and malate were linear mixed-type inhibitors, whereas 6-phosphogluconate was a linear non-competitive inhibitor for the cleavage reaction catalysed by Phycomyces isocitrate lyase. Inhibition by malate has been also reported for the enzymes from Ps. indigofera enzyme [53] (uncompetitive), N. crassa [9] (non-competitive) and Br. flavum [50] (competitive), whereas isocitrate lyases from Candida tropicalis [51] and Rhodotorula [54] were inhibited by 6-phosphogluconate.

As shown in Table 3, itaconate and maleate, two compounds without physiological significance, were the most effective inhibitors of the cleavage reaction catalysed by Phycomyces isocitrate lyase. Iaconate is also an uncompetitive inhibitor for isocitrate lyases from Ps. indigofera [30], Lupinus [55] and Pinus densiflora [29] and a non-competitive inhibitor for the Linum usitatissimum enzyme [48] and the water-melon cotyledon enzyme [42]. Inhibition by phosphoenolpyruvate has been observed in all isocitrate lyases studied (except for the Brevibacterium enzyme) [26], it being suggested that its effects might be physiologically significant, although recent studies on isocitrate lyase from E. coli argued against this possibility [6], at least for the enzyme from this enteric bacterium. The results reported in the present paper indicate that the activity of the isocitrate lyase from Phycomyces blakesleanus could be regulated in vivo by, in general, the tricarboxylic acid-cycle and/or glycolytic intermediates, which are replenished by the anaplerotic glyoxylate cycle. However, an estimation of intracellular concentrations of these intermediates is necessary in order for it to be concluded that changes in their concentrations have physiological impact.

On the other hand, in the regulation of isocitrate lyase the isocitrate dehydrogenase activity may play an important role. Experiments in our laboratory (M. J. Caloca, P. del Valle, D. de Arriaga, F. Busto & J. Soler, unpublished work) allow us to indicate that the competition between the glyoxylate by-pass and the tricarboxylic acid cycle at the level of isocitrate may be resolved, at least, by changes in the allosteric properties of the NAD-dependent isocitrate dehydrogenase activity. It is also noticeable that the magnesium isocitrate complex is the true substrate for this isocitrate dehydrogenase activity also.

### Table 3. Inhibition of Ph. blakesleanus isocitrate lyase

<table>
<thead>
<tr>
<th>Compound</th>
<th>Type of inhibition</th>
<th>Inhibition constant ($K_i$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Cleavage reaction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Succinate</td>
<td>Linear mixed-type</td>
<td>1.3 ± 0.4 mM</td>
</tr>
<tr>
<td>Phosphoenolpyruvate</td>
<td>Linear non-competitive</td>
<td>0.63 ± 0.03 mM</td>
</tr>
<tr>
<td>Itaconate</td>
<td>Linear uncompetitive</td>
<td>5.9 ± 0.5 μM</td>
</tr>
<tr>
<td>Oxalate</td>
<td>Linear competitive</td>
<td>1.4 ± 0.1 mM</td>
</tr>
<tr>
<td>Fumarate</td>
<td>Linear competitive</td>
<td>4.8 ± 1.0 mM</td>
</tr>
<tr>
<td>Maleate</td>
<td>Linear uncompetitive</td>
<td>0.2 ± 0.02 mM</td>
</tr>
<tr>
<td>6-Phosphogluconate</td>
<td>Linear non-competitive</td>
<td>1.3 ± 0.3 mM</td>
</tr>
<tr>
<td>Citrate</td>
<td>Linear mixed-type</td>
<td>1.9 ± 0.1 mM</td>
</tr>
<tr>
<td>Malate</td>
<td>Linear mixed-type</td>
<td>22.0 ± 0.3 mM</td>
</tr>
<tr>
<td>(b) Condensation reaction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inhibitor versus glyoxylate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphoenolpyruvate</td>
<td>Linear uncompetitive</td>
<td>0.83 ± 0.15 mM</td>
</tr>
<tr>
<td>Itaconate</td>
<td>Linear non-competitive</td>
<td>4.7 ± 0.3 μM</td>
</tr>
<tr>
<td>Oxalate</td>
<td>Linear competitive</td>
<td>51.0 ± 4 μM</td>
</tr>
<tr>
<td>Inhibitor versus succinate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphoenolpyruvate</td>
<td>Linear competitive</td>
<td>0.72 ± 0.09 mM</td>
</tr>
<tr>
<td>Itaconate</td>
<td>Linear competitive</td>
<td>5.2 ± 0.7 μM</td>
</tr>
<tr>
<td>Oxalate</td>
<td>Linear mixed-type</td>
<td>0.12 ± 0.01 mM</td>
</tr>
</tbody>
</table>

**Fig. 7. Thiol groups in Ph. blakesleanus isocitrate lyase**

The purified enzyme was treated in the native state, or after denaturation with 5,5'-dithiobis-(2-nitrobenzoic acid), for thiol group determination as described in the Experimental section. The reaction was performed at different isocitrate lyase concentrations in 25 mM-imidazole/HCl buffer, pH 6.8, at 30 °C in a total volume of 1 ml. The number of thiol groups per subunit (M, 62000) was calculated to be 2.89 for the denatured enzyme (○) and 1.84 for the native enzyme (●).
isocitrate lyase [10] but lower than that shown for isocitrate lyase from *Ps. indigofera*, which was 19–21, and for that from *N. crassa*, which was 33 [9,32,57]. Chell et al. [10] claim a more thermostable structure in view of the relative paucity of thiol groups in the thermophilic *Bacillus* isocitrate lyase in comparison with the *Pseudomonas* and *Neurospora* enzymes, but our results for the *Phycycomyces* enzyme with the same number of thiol groups cannot support this suggestion, in view of the temperature-instability of this enzyme in the absence of denaturant agents. The formation of 5-mercaptop-2-nitrobenzoate at pH 6.8 from 0.1 mM-5,5'-dithiobis(2-nitrobenzoic acid) follows biphasic kinetics (results not shown). Extrapolation of the reaction trace in the slow phase to the ordinate intercept of the semi-logarithmic plot of $A_{\infty} - A$ versus time and a replot of the fast-phase reaction data shows that 1 equiv. of 5-mercapto-2-nitrobenzoate is formed in each phase. This result is consistent with the titration of two distinct thiol groups per subunit. $A_{\infty}$ and $A_{s}$ represent changes in absorbance at 412 nm at a given time and at 100% reaction respectively.

**Inactivation by iodoacetate**

Isocitrate lyase was inactivated by iodoacetate in a time- and concentration-dependent manner at pH 6.8 and 25 °C (Fig. 8). No detectable activity remained after prolonged incubation. A semi-logarithmic plot of residual activity against time was not linear, and a rapid decrease in activity was followed by a slower reaction that led to total loss of activity.

The kinetics of inactivation shown in Fig. 8 suggests that isocitrate lyase from *Phycycomyces* has two classes of modifiable thiol groups, the alkylation of which has different effects on enzyme activity. However, an alternative notion could be that it represented a conformational change in the protein rather than further substitution. The slow phase of the inactivation was apparently linear, dependent on time but not on the concentration of iodoacetate used, and had a first-order rate constant of 0.018 min⁻¹. Extrapolation of this phase to the zero-time axis yielded a value of about 2.4% of the initial velocity for 5 mM-iodoacetate. At a given time, subtraction of the values obtained by extrapolation from the observed values of remaining activity gives a straight line with a slope from which $k_{app}$ may be determined [58]. For the fast-reacting thiol groups, $k_{app}$ is the pseudo-first-order rate constant. In the range of concentration tested, reaction of iodoacetate with the fast-reacting thiol groups was shown to follow an apparent hyperbolic behaviour (Fig. 9a). This could indicate that the inactivation process involves the initial formation of a non-covalent enzyme–modifier complex [59]. From a plot of [iodoacetate]/$k_{app}$ versus [iodoacetate] (Fig. 9b) we have obtained a limiting rate constant of inactivation of 0.83 min⁻¹. The dissociation constant for the enzyme–iodoacetate complex as calculated from this plot was found to be 3.15 mM. This result suggests that the iodoacetate interacts specifically with the fast-reacting thiol groups at a site on the enzyme (presumably the active site).

Isocitrate and Mg²⁺ partially protected the *Phycycomyces* isocitrate lyase against iodoacetate modification. Whichever ligand was used, non-linear semi-logarithmic plots were obtained, indicating the involvement of both sets of thiol groups in the inactivation reaction. The slowly reacting groups were unaffected by the presence of ligands, which may indicate that these slowly reacting groups did not belong to the active site.

Our results of protection against inactivation, obtained for 5 mM-iodoacetate, were analysed by plots of $\Delta k$ versus [ligand] according to the partial protection model described by Renosto et al. [60].

Isocitrate lyase displayed hyperbolic plots of $\Delta k$ versus [Mg²⁺]. From a double-reciprocal plot of $1/\Delta k$ versus $1/[Mg^{2+}]$ we calculated a value of $\Delta k_{max(app)}$ of 0.42 min⁻¹. In the absence of

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**Fig. 8. Inactivation of *Ph. blakesleeanus* isocitrate lyase by iodoacetate**

Isocitrate lyase (0.83 μM) was incubated with iodoacetate at the concentrations indicated as described in the Experimental section. At a given time, the values of the residual activity for each iodoacetate concentration corresponding to the reaction with the slowly reacting thiol groups were extrapolated and subtracted from the remaining activity observed. The slopes of the resulting lines were used to determine the pseudo-first-order rate constant. Line $A$ represents the corrected values for 5 mM-iodoacetate.

**Fig. 9. Influence of iodoacetate concentrations on the pseudo-first-order rate constant of the fast-reacting thiol groups in the inactivation of *Ph. blakesleeanus* isocitrate lyase**

Data for $k_{app}$ are the corrected values obtained from Fig. 8, including the $k_{app}$ value obtained for 0.25 mM-iodoacetate. (a) Direct representation and (b) plot of [iodoacetate]/$k_{app}$ versus [iodoacetate].
Mg$^{2+}$ the $k_{\text{app}}$ value was 0.55 min$^{-1}$, and thus the limiting inactivation constant in the presence of Mg$^{2+}$ was 0.13 min$^{-1}$. That is, the enzyme--Mg$^{2+}$ complex has less susceptibility to the iodoacetate inactivation, resulting mainly from a decrease in the reactivity of this thiol group.

On the other hand, the isocitrate lyase displays sigmoidal plots of $A_A$ versus [isocitrate] with an $n$ value of 1.6 with a value for $D_A$-isocitrate concentration that gave a 50% protection of 4–5 nm. The sigmoidicity shown in the protection by isocitrate may be indicative that free isocitrate binds co-operatively to the isocitrate lyase from Ph. blakesleeanus.

Our results are in agreement with data reported for isocitrate lyases from other sources such as N. crassa, Ps. indigofera, watermelon cotyledons and E. coli [14,35,42,57], which show the existence of a cysteine residue at the active site. However, more detailed studies will be required to test the identity of the thiol-containing amino acid residue, the subite to which it can be assigned and the effects of iodoacetate modification on the regulation of isocitrate lyase activity.

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REFERENCES


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