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ISOCITRATE LYASE FROM HIGHER PLANTS*

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Key Word Index: Isocitrate lyase; higher plants; structure and catalysis; glyoxylate cycle; review.

Abstract—Work on isocitrate lyase, the first enzyme unique to the glyoxylate cycle, is reviewed.

INTRODUCTION

Isocitrate lyase (ICL,§ *threo*-D_s-isocitrate-glyoxylate lyase, EC 4.1.3.1), the first enzyme unique to the metabolic pathway known as the glyoxylate cycle, reversibly catalyses the cleavage of isocitrate into succinate and glyoxylate according to the reaction:

$$\text{isocitrate} \rightleftarrows \text{succinate} + \text{glyoxylate} \quad \text{(I)}.$$

In the past few years, so many papers on ICL from higher plants have been published that we think it appropriate to review them at least briefly.

Discovery, function, and occurrence

The enzyme was discovered by Campbell *et al.* [1] in *Pseudomonas aeruginosa*. Some years later, Kornberg and Beevers [2] demonstrated the presence and role of ICL in germinating castor beans. The function of the glyoxylate cycle in the conversion of fats to carbohydrates in fat-storing and oil-rich seeds soon became evident (for a review, see ref. [3]). The glyoxylate cycle is widely distributed among plants. It is worth noting that ICL alone, in the absence of malate synthase, has been reported as occurring in the leaves of *Pisum sativum* [4], *Spinacia oleracea*, *Triticum aestivum*, and *Zea mays* [5], and in cell cultures from *Rosa* [6]. Several species in which the glyoxylate cycle operates are listed in Table 1.

Table 1. Distribution of isocitrate lyase and glyoxylate cycle among higher plants

Taxonomic name	Common name	References
Gymnosperms		
Abies alba	fir	[67]
Gingko biloba	gingko	[68]
Pinus canariensis		[67]
P. densiflora		[28]
P. palustris	longleaf pine	[69]
P. pinaster		[67]
P. pinea		[67]
P. ponderosa	ponderosa pine	[70]
P. strobus		[67]
Angiosperms		
Arachis hypogaea	peanut	[71]
Borago officinalis	borage	[72]
Brassica napus	rapeseed	[73]
Corylus avellana	hazel	[74]
Cucumis citrullus	watermelon	[71]
C. sativus	cucumber	[75]
Cucurbita maxima	squash	[76]
C. pepo	pumpkin	[71]
	marrow	[77]
Glycine max	soybean	[71]
Gossypium hirsutum	cotton	[71]
Helianthus annuus	sunflower	[78]
Linum usitatissimum	flax	[71]
Lupinus alba	lupin(e)	[79]
Musa cavendishii	banana (fruit)	[80]
Olea europaea	olive	[81]
Oxalis pescaprae	sorrel	[82]
Persea gratissima	avocado (fruit)	[71]
Pimpinella anisum	anise	[83]
Ricinus communis	castorbean	[84]
Sesamum indicum	sesame	[85]
Simmondsia chinensis	jojoba	[86]
Sinapis alba	mustard	[87]
Triticum vulgare	wheat	[88]
Zea mays	maize	[89]

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§*Abbreviations used*: BrP, bromopyruvate; DEAE-, diethylaminoethyl-; DTE, 1,4-dithioerythritol; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetate; GSH, reduced glutathione; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ICL, isocitrate lyase; MOPS, morpholinepropanesulfonic acid; PEP, phosphoenolpyruvate; 3P-GA, 3-phosphoglycerate; PMSF, phenylmethylsulfonylfluoride; TRA, triethanolamine; Tris, Tris-(hydroxymethyl)-aminomethane.

Intracellular localization

As with all the enzymes of the glyoxylate cycle, including the three isozymes of Krebs cycle enzymes (malic dehydrogenase, citrate synthase, aconitase), ICL is localized in glyoxysomes [3]. A scheme of the glyoxylate cycle and its probable relation to the tricarboxylic acid cycle and other correlated pathways in germinating seeds are shown in Fig. 1. Within the glyoxysomes, ICL is commonly found in the matrix [3]. In pea leaves [4] and in rose [6] ICL is reported to be localized in mitochondria.

Biosynthesis

Opinions differ about whether ICL-*m*RNA exists before germination [7–9] or whether it is newly transcribed [10–13]. The enzyme levels in the cotyledons or endosperm of germinating seeds have similar time-courses. ICL is synthesized *de novo*, increases to a maximum and declines to a negligible value. The time of the maximal-activity peak seems to be a characteristic of each species. For example, in the dark it is about 3 days for *Linum usitatissimum*, *Cucurbita pepo*, *Cucumis sativus*, *Gossypium hirsutum*, and *Citrullus vulgaris*, 3–4 days for *Helianthus annuus*, 4–5 for *Ricinus communis*; 7 for *Arachis hypogaea*; 11 for *Lupinus* and 10–14 days for various *Pinus* species. The amount and developmental pattern of the enzyme activity may vary depending on the germination conditions, such as temperature, light, hormones, and metabolites [3]. The biogenesis of glyoxysomes has recently been reviewed in ref. [14].

ISOLATION OF ISOCITRATE LYASE

Assay of enzyme activity

The methods used for assaying ICL activity may be coupled both chemically and enzymatically. Among chemical methods, two assays, one discontinuous [15, 16] and the other continuous [17], are available. The enzymatic methods are coupled with lactate dehydrogenase in the direction of isocitrate cleavage [18] or with NADP-isocitric dehydrogenase in the opposite direction [19, 20].

Isolation procedure

ICL has been purified to homogeneity from only a few plant sources: *Linum usitatissimum* [21], *Cucumis sativus* [22, 23], *Ricinus communis* [24], *Citrullus vulgaris* [25], *Gossypium hirsutum* [26], *Pinus pinea* [27], and *Pinus densiflora* [28]. Purifications to a lower degree are reported from *Helianthus annuus* [29] and *Lupinus* [30].

The procedures for isolating ICL from higher plants are quite similar. The protocol often consists in extraction, heat treatment, ammonium sulphate fractionation and gel filtration. The final step is usually carried out by anion-exchange techniques. Pinzauti *et al.* [27], in a recent improved procedure, have obtained very good results with hydrophobic interaction chromatography on octyl-Sepharose which allows the complete resolution of *Pinus* ICL from catalase, a frequent contaminant of other final preparations [22, 23, 31]. Some procedures start with purified glyoxysomes and include the separation of glyoxysomal matrix enzyme by zonal centrifugation [23].

The specific activities of pure preparations range between 20 units/mg of protein for *L. usitatissimum* [21] and 2.7 for *R. communis* [24]. The percentages of ICL per total extractable proteins are about 0.6–1.5%. The best final yield (about 36%) is achieved with *C. vulgaris*, probably because of the lower instability of the ICL from this source [25].

STRUCTURAL FEATURES

Primary structure

Amino acid composition. The amino acid composition of higher plant ICL has so far been determined for *Linum usitatissimum* [32], *Citrullus vulgaris* [25], and *Pinus pinea* [27]. The values are listed in Table 2. The analysis of ref. [33] indicates that the enzymes are closely related, being SΔQ less than 50 units in all cases: i.e. 9, 21, 40 SΔQ units for comparisons between pine–watermelon, pine–flax and flax–watermelon, respectively.

Charge and isoelectric point. Most plant ICLs appear to be poorly charged at pH 7.0–7.7, as shown by their behaviour in ion-exchange chromatography. *Ricinus* [24], *Pinus* [34], and *Citrullus* [25] enzymes are not retained by DEAE-exchangers, while *Cucumis* ICL [22, 23] is eluted at a low salt concentration (0.050 M). Only *Linum* [46] and *Lupinus* [Vanni, unpublished data] ICLs are eluted at higher salt concentrations: 0.142 and 0.100 M, respectively. The ratio (Asx + Glx) to (Lys + His + Arg) in the available amino acid compositions is higher than unity, consistent with the enzymes' low pI. *Linum* ICL, which also binds to DEAE-cellulose, has the smallest ratio.

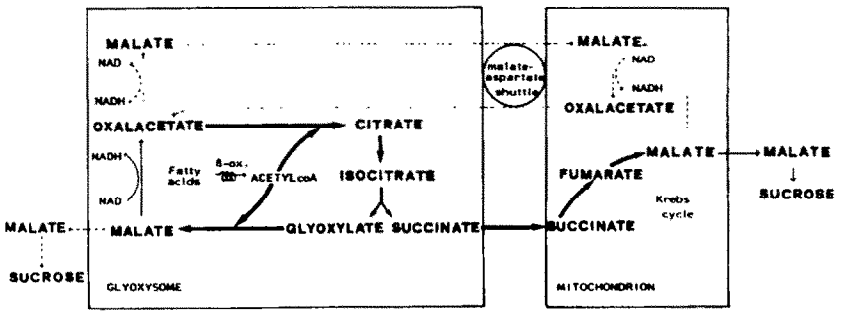


Fig. 1. Relations between the glyoxylate cycle and Krebs cycle in higher plants. The dashed lines, alternative to the continuous thin lines, show a proposed malate–aspartate shuttle for the transfer of reducing equivalents between glyoxysomes and mitochondria [90].

Table 2. Amino acid composition* of isocitrate lyase from higher plants

Amino acid	*Pinus pinea* (1)	*Linum usitatissimum* (2)	*Citrullus vulgaris* (3)
Asx	229	224	227
Thr	151	133	179
Ser	141	105	175
Glx	264	262	285
Pro	84	62	80
Gly	181	214	173
Ala	268	292	265
Cys	24	28	37
Val	151	219	128
Met	89	61	49
Ile	95	108	118
Leu	189	160	194
Tyr	69	90	72
Phe	96	101	105
Lys	144	143	144
His	61	60	65
Arg	142	117	164
Trp	18	31	32
Total	2396	2410	2492

*Number of residues (nearest integer per oligomer) obtained as follows: (1) data from Pinzauti *et al.* [27]; (2) recalculated from Khan and McFadden [26]; (3) recalculated from Jameel *et al.* [25].

The isoelectric point of *Cucumis* enzyme is 5.9, a secondary form has a value of 6.4 [23]. In urea, the pI of this ICL is 5.1 [22]. The isoelectric point of *P. pinea* enzyme was previously found to be 7.2 [27]. A more accurate measurement technique—combined electrofocusing–electrophoresis in polyacrylamide gel [35]—has now shown *Pinus* ICL to have a real pI of 6.3 and a very poor electrophoretic mobility between pH 5 and 9, which accounts for its difficult focusing with conventional methods. Moreover, the strong interaction with octyl-Sepharose [27], indicates that *P. pinea* ICL is highly hydrophobic. This enzyme also binds to phenyl-Sepharose [27], whereas *Cucumis* (?) ICL does not [36].

Terminal amino acids. Among higher plants, only *C*- and *N*-terminals of *L. usitatissimum* ICL have been identified [32]. The *C*-terminal is histidine, with phenylalanine in the penultimate position; the *N*-terminal is methionine. These findings resemble those obtained with other sources (*Pseudomonas indigogera* [37] and *Neurospora crassa* [38]), with the exception of *Bacillus* enzyme [39]. Attempts to determine the terminal residues of *Pinus pinea* enzyme [27] have proved unsuccessful, probably because there are technical problems, since the material becomes unsoluble during the experimental procedure.

Sugar content. There is no agreement as to whether or not ICLs from various sources have a sugar moiety. Frevert and Kindl [23] reported that *Cucumis* enzyme is a glycoprotein, but their observation was subsequently contested by Riezman *et al.* [40] who, using gas chromatography, found no evidence of sugar content in the enzyme from the same source. The latter finding seems to be confirmed by ref. [41] for *Ricinus communis*, ref. [25] for *Citrullus vulgaris*, and ref. [27] for *Pinus pinea*.

Tertiary and quaternary structure

All ICLs consist of four subunits except for *P. densiflora* and *Cucumis* enzymes which are reported to be a trimer [28] and a pentamer [22], respectively. The latter observation is contradicted by Frevert and Kindl [23] who described *Cucumis* ICL in its usual tetrameric conformation. The subunits appear to be identical in size. The subunit and oligomer molecular weights and the Stoke's radia of plant ICLs are reported in Table 3. Some of the discrepancies may be due to the different techniques of evaluation. In any case, the values are homogeneously distributed between 62000 and 67000, with the only striking exception of sunflower ICL whose monomer has a M_r of 28500 [29]. The low figure for *Ricinus* ICL [24] contrasts with the value of 62000 reported in ref. [42] for the enzyme monomer from the same source. The possibility of proteolytic cleavage during ICL preparation has often been suggested [21, 25, 31, 34].

Few data are available about quaternary structure of plant ICL. The minimum quaternary structure necessary for catalysis seems to be the tetramer. The forces involved in maintaining the quaternary conformation are unknown. In experiments of labelled-oxalate binding with *Pinus* ICL, Pinzauti *et al.* [27] found four independent catalytic sites per oligomer. Malhotra and Srivastava [24] suggested that *Ricinus* ICL has a twofold axis of symmetry, since its heat-inactivation shows a characteristic biphasic kinetic. We think that their data are not sufficient to exclude the presence of altered enzyme forms differing in thermal-stability.

Table 3. Molecular weights, subunit number and Stoke's radii of higher plant ICLs

Source	Subunit M_r	No. sub.	Oligomer M_r	Stoke's radius (nm)	References
Citrullus vulgaris	64000	4	277000		[25]
Cucumis sativus	64000	4	255000	5.50	[23]
	63500	5	325000		[22]
Helianthus annuus	28500	4	114000		[29]
Linum usitatissimum	67000	4	264000	5.28	[21]
Lupinus	66000	4	260000	5.37	[30]
Ricinus communis	35000	4	140000		[24]
	62000				[42]
Pinus pinea	66000	4	264000	5.46	[27]
Pinus densiflora	65000	3	200000		[28]

Multiple forms

Multiple forms of ICL are frequently reported in enzyme purifications from higher plants. These forms probably arise from proteolytic modifications after cell rupture, but they might also be due to complex mechanisms which lead to ICL decline *in vivo*. Khan *et al.* [21] described two ICL forms from *Linum usitatissimum*, having the same molecular weight but differing in electric charge and specific activity. Two years later, the same group [9] provided evidence for the presence of a low M_r (20000–25000) endopeptidase inhibited by PMSF and responsible for the selective inactivation of ICL. The protease appeared to be synthesized at advanced germination. Theimer [43] had previously reported the presence of a proteinaceous factor which selectively inactivated ICL in homogenates from *Helianthus annuus* cotyledons. Multiple ICL forms differing in electric charge [22] or pI [23] were observed in *Cucumis sativus*. Forms differing in M_r, both *in vivo* (monomer M_r 63000 and 61500) and after RNA translation *in vitro* (61500 and 60000), were subsequently described from the same source, probably reflecting the processing of ICL necessary for its transport across glyoxysomal membranes [40, 44].

Following the observation of multiple ICL forms in *Lupinus* [45] and *Pinus pinea* [31], Pinzauti *et al.* [34] demonstrated that this multiplicity is related, at least in part, to the stage of germination. Even if proteolysis after cell rupture plays a role in generating multiple forms, the authors suggested that an alteration of the native enzyme was presumably occurring *in vivo*.

A correlation between protease action and ICL instability has been demonstrated in castor bean endosperm [46] and flax seedling [47] homogenates. Jameel *et al.* [48] provided a further demonstration of proteolytic activities related to germination in crude extracts from *Linum* seeds, and they too suggested a possible correlation with ICL loss *in vivo*. Two ICL-active components (one of which predominates) were observed by conventional gel electrophoresis in a pure enzyme from *Citrullus vulgaris* [25]. The same preparation appeared to consist of a heat-stable (about 60%) and a heat-sensitive fraction. Recently, Pinzauti *et al.* [27] demonstrated that charge and size heterogeneities can be eliminated by shortening the time of seed germination and by the use of PMSF during the whole purification procedure.

CATALYTIC FEATURES

Thermodynamics

The equilibrium constant for isocitrate formation by succinate and glyoxylate condensation was first estimated from studies on bacterial ICLs [49, 50], and only later on *Pinus pinea* and *Lupinus* enzyme. The values, as determined by Haldane's treatment (pH 7.5, 30°), were found to be 796 M^{-1} (which corresponds to a free-energy change of −4.0 kcal/mol) with *P. pinea* ICL [31], and 980 M^{-1} with *Lupinus* enzyme [30]. These figures agree with the previously reported values of ref. [50] but contrast with those of ref. [49] (for a discussion see ref. [50]).

The activation energy was found to be 8.7 kcal/mol for *Linum usitatissimum* ICL [21]. The complete set of ICL activation parameters has never been reported for higher plant enzyme. No data exist on the activation parameters for the various stages of the catalysed reaction, including those on the formation of the enzyme-substrate complexes. Under standard conditions the reaction catalysed by ICL is spontaneous in the direction opposite to the physiological one as established in ref. [49] on bacterial enzyme. In any case, the glyoxylate formed by the ICL reaction is removed by the malate synthase reaction whose $\Delta G°$ is about −12 kcal/mol. We know of no physiological conditions under which ICL acts to catalyse the condensation of glyoxylate and succinate to isocitrate.

Specificity

All ICLs are specific for only one of the four possible isocitrate isomers, namely *threo*-D_s-isocitrate*. As was first shown by Olson [51] for *Penicillium chrysogenum* ICL and later for *Pinus pinea* enzyme [31; Vanni *et al.*, unpublished], ICLs are not inhibited by *threo*-L_s-isomer. Malhotra *et al.* [52] have recently reported that *threo*-D_s-isocitrate lactone can by cleaved by *Ricinus* ICL at a rate which is about 30% of that for *threo*-D_s-isocitrate. This finding contrasts with all the others reported to date; in fact, isocitrate lactone has always been found to be ineffective as a substrate of ICL [53]. A high specificity has also been found for glyoxylate and succinate by studies on bacterial ICL [53], but no data are available for higher plant enzyme.

Requirement for cofactors

Effect of magnesium. All ICLs require Mg^{2+} for activity [53]. There is no evidence that ICL contains a coenzyme. The optimal Mg^{2+} concentrations have been found to be 6 mM for *P. pinea* [31], 5 mM for *Lupinus* [45], 3.7 mM for *L. usitatissimum* [21], 5–6 mM for *C. sativus* [23], and 3 mM for *P. densiflora* [28] ICLs. Higher concentrations have an inhibitory effect. From studies on bacterial ICL, Mg^{2+} has been assumed to be bound by the free enzyme to give the active enzyme form which binds the substrates [53]. As is the case with other Mg-dependent enzymes [54], the true substrate might also be the Mg-substrate complex. It is not clear at present which of these represents the true mechanism for ICL reaction. Moreover, other models are also possible [55]. Studies on *P. pinea* enzyme indicated that Mg^{2+} acts as a protective agent against thermal inactivation, even in the absence of any substrate [34, 28]. Moreover, in experiments with *Pinus* ICL exhaustively dialysed against EDTA, Mg ions were found to be tightly bound to the enzyme [Vanni *et al.*, unpublished]. These findings suggest that a large fraction of ICL is probably present in an Mg-bound state.

Malhotra *et al.* [56] explained the apparent negative cooperativity, which they observed in *Ricinus* ICL when total Mg^{2+} concentration increases, by suggesting the existence of two classes of site for Mg^{2+} with different affinities. It is to be noted that in this study the authors assumed the free Mg^{2+} to be equal to the total Mg^{2+} added to the assay mixture, neglecting the complex interactions existent among ICL, substrates and Mg ions.

*In the past the isocitrate-isomer nomenclature differed from that proposed by Vickery [91] and universally accepted now. According to Cahn, Ingold and Prelog's notation [92], which eliminate any misunderstanding, the substrate of ICL is (1*R*, 2*S*)-1-hydroxy-1,2,3-propanetricarboxylic acid [93].

Effect of other divalent cations. In *L. usitatissimum* ICL, Co^{2+} and Mn^{2+} can substitute for Mg^{2+} with an efficiency of 29% and 24%, respectively [21]. Among divalent cations, only Ba^{2+} and Ca^{2+} can replace Mg^{2+} in ICL from *P. densiflora* though with very low efficiencies (11% and 7%, respectively) [28]. Several divalent cations—Ba^{2+}, Ca^{2+}, Ni^{2+}, Mn^{2+}, Co^{2+}, in increasing order—inhibit *P. pinea* ICL in the presence of Mg^{2+} [31]. A relationship seems to exist between ionic radius and inhibitory effect, since the cations with ionic radia close to that of Mg^{2+} are stronger inhibitors. Heavy metal ions are strong inhibitors of *P. pinea* [31] and *P. densiflora* [28] ICLs.

Effect of inorganic anions. Few data are available for higher plant ICL [53]. The HPO_4^{2-} ion is a powerful inhibitor of the enzyme from *L. usitatissimum* [21], *Lupinus* [45], and *P. pinea* [27]. SO_4^{2-} inhibits ICL from *C. sativus* [23].

Effect of –SH *reagents.* All ICLs can be (re)activated by several –SH compounds, such as 2-mercaptoethanol, GSH, cysteine, DTT, DTE [53], except *P. densiflora* enzyme which is inhibited by these compounds [28]. The major function of –SH compounds is thought to be that of maintaining in the reduced state one or more –SH groups of cysteine residues which probably are part of the active site and involved in the catalytic mechanism of ICL [53]. In fact some –SH-directed modifiers, such as *p*-chloromercuribenzoate [45, 28] and *N*-ethylmaleimide [57] exert an inactivating effect. Evidence for an –SH group involvement in the catalytic mechanism of ICL was obtained via active-site affinity labelling (see below). –SH compounds seem to be unable to prevent the decay of *P. pinea* enzyme activity during storage, and the reactivation of old enzyme preparations is time dependent: a 6-day-old pure fraction can be reactivated up to 50%, but no effect is obtained in a 14-old-old one [58]. 2-Mercaptoethanol (2 mM) has been found to accelerate inactivation of *P. densiflora* ICL [28].

Stability

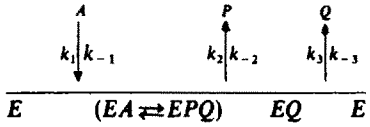
Most ICLs are very unstable. In general, higher plant ICLs are more unstable than the bacterial enzyme [25, 28, 31, 45, 59] and the lower the storage temperature, the longer is ICL activity maintained [58, 25]. This characteristic, while recognized, has not been sufficiently emphasized in the literature, probably because it was considered just a technical aspect of enzyme storage. Indeed, ICL instability may often be correlated with the action of proteolytic enzymes [31, 46–48] or of a specific modifiers [43], both of which may play a role in the physiological regulation of the enzyme. In this view, the ICL thermal-sensitivity has a special significance, as Dreyfus *et al.* [60] pointed out for the aging of enzymes. Mg ions [34, 28] or oxalate [25] provide protection against thermal inactivation. ICL forms differing in thermal sensitivity have been observed in *Pinus* [34], and *Citrullus* [25]. ICL from *Pinus* species has also been found to be highly unstable at low pH and high pH [Vanni *et al.*, unpublished; 28]. In the absence of Mg^{2+}, *Pinus* ICLs become extremely unstable: 50% of initial activity overnight at 0° [28], or after 5 hr at 30° [Vanni *et al.*, to be published].

Table 4. Rate constants for ICL from *Lupinus*

Rate constant	(s^{-1})	Rate constant	($M^{-1} s^{-1}$)
k_{-1}	14.5	k_1	725000
k_2	100.5	k_{-2}	191700
k_3	11.6	k_{-3}	145000

Kinetic studies

Kinetic mechanism. ICL kinetic mechanism was initially studied by steady-state kinetic analysis in the bacterium *P. indigofera* [50]. The kinetic mechanism of plant ICLs was studied some years later in *L. usitatissimum* [32], *P. pinea* [31], *Citrullus vulgaris* [61], and *Lupinus* [30]. All the data support a sequential, ordered, uni–bi/bi–uni catalytic mechanism in which succinate is the first product to be released (or the second substrate to enter the active site). The reaction scheme, according to Cleland's nomenclature, is reported below.



The ICL-glyoxylate complex represents the largest enzyme-form at any isocitrate concentration [30]. Similar results had already been reported by Williams *et al.* [50] for *P. indigofera* ICL. Typical rate costants of the ICL reaction for *Lupinus* enzyme [30] are given in Table 4.

McFadden and co-workers [32] reported that ICL should be considered as a random system with a largely preferred pathway so that in standard kinetic analysis the kinetic mechanism is practically ordered. Assuming an equilibrium model and not taking into account the second substrate, Malhotra *et al.* [52] have reported that *Ricinus* ICL has a random kinetic mechanism. Daron *et al.* [62] first pointed out that the kinetic analysis data may account for either an ordered mechanism under steady state conditions or a random mechanism under equilibrium conditions.

Some catalytic properties of various ICLs are listed in Table 5. In comparing K_m values, one should bear in mind that the buffering system affects ICL kinetics (e.g. phosphate ion is a competitive inhibitor, as reported above) and that the K_m of ICL is pH-dependent (see below).

Cooperativity. ICL from *Chlorella vulgaris* was early reported to exhibit non linear kinetics [63], but cooperativity has never been found in later studies. In *Ricinus* ICL, Malhotra *et al.* [52] observed a negative cooperativity for the binding of both glyoxylate and succinate which they explained in terms of the existence of two classes of binding sites for both succinate and glyoxylate with different affinities ('tight' and 'loose' sites).

Inhibition of ICL. The effect of various metabolites on several ICLs is shown in Table 6 along with the type of inhibition with respect to isocitrate, when available. The inhibition by glyoxylate and succinate is explained by the reaction mechanism discussed earlier. Other metabolites act by a product–analogue mechanism. This is the case for oxalate and glycolate (glyoxylate analogues) and itaconate and PEP (succinate analogues).

Substrate inhibition. ICL substrate-inhibition in the condensation reaction was reported for *Lupinus* [57] and

Table 5. Catalytic properties of higher plant ICLs

Source	K_m isocitrate (mM)	Molar activity* (mol isocitrate/sec/ mol active site)	pH optimum	References
Citrullus vulgaris	0.250 (MOPS, pH 7.5)	18.6	—	[25]
Cucumis sativus	0.039 (MOPS, pH 6.8)	6.3	6.75 MOPS	[22]
	0.100 (HEPES, pH 7.4)	7.6	7.2–7.6 HEPES	[23]
Helianthus annuus	0.032 (phosphate, pH 6.0)	2.2	7.5 phosphate	
	0.667 (phosphate, pH 7.5)		7.35 imidazole	
			7.6 Tris	[29]
Linum usitatissimum	0.289 (phosphate, pH 7.5)	22.0	7.5 Tris or phosphate	[21]
Lupinus	0.035 (imidazole, pH 7.0)	2.0	6.8 imidazole	[30, 31]
Ricinus communis	0.300 (phosphate, pH 7.0)	1.6	7.2–7.3 phosphate	[24, 52]
	1.000 (phosphate, pH 6.8)			
Pinus densiflora	0.660 (Tris, pH 7.6)	5.3	7.6 Tris	[28]
Pinus pinea	0.033 (HEPES, pH 7.0)	11.1	7.3 HEPES	
	0.110 (TRA, pH 7.0)		7.3 TRA	
			7.5 imidazole	[27, 31]

*Calculated from the specific activities of the purified enzymes and the oligomer M_r reported in Table 3.

Table 6. Inhibitory effect by various metabolites on higher plant ICLs

	Citrullus vulgaris [61]	*Helianthus annuus* [29]	*Linum usitatissimum* [21, 32]	*Lupinus* [30, 45]	*Ricinus communis* [52]	*Pinus densiflora* [28]	*Pinus pinea* [27, 31]
ADP						+	
ATP						+	
cis-Aconitate		C					
Citrate		C			NC		
Glycolate		C		C			C
Glyoxylate			C	C			C
Itaconate	NC		NC	UC		UC	UC
Malate				+	NC		
Maleate					NC	+	
Malonate	C		C	+	NC		
Oxalate	C	C		C		+	C
PEP			NC	+		+	
3-PGA						+	
Succinate	NC	C	NC	NC	C		NC
Tartrate	C		C	+		+	

C = competitive, NC = non-competitive, UC = uncompetitive.

P. pinea [31] enzymes. Malhotra *et al.* [52] have described a complex substrate inhibition in the cleavage reaction for *Ricinus* ICL in which isocitrate was found to be a substrate inhibitor at pH 6 and pH 8, but not at pH 6.8.

pH dependence of V_{max} *and* K_m. There are some discrepancies in pH optimum patterns related to the buffer used. Differences of 0.5 and of 1 pH unit were found for Lupinus ICL [45] and for cucumber enzyme [22], respectively. In imidazole buffer, a sharp difference between the pH optima for cleavage and condensation reactions was found in *Lupinus* ICL [57] and, subsequently, in *P. pinea* ICL [31] and *Helianthus annuus* [29]. No such difference, however, was found for *P. pinea* in Hepes and TRA buffers [31]. Because of substrate inhibition in the condensation reaction [57, 31], unsaturating concentrations of glyoxylate and succinate were used in determining pH optima for *Lupinus* [57] and *P. pinea* [31] ICLs. As a result, some of the discrepancies observed between cleavage and condensation reactions may be due to the fact that the true effect of pH upon V_{max} is not measured since under unsaturating conditions the results are affected by the K_m dependence upon pH. The effect of pH upon K_m was studied in *P. pinea* ICL [31]. Since the function of pK_m versus pH between pH 6.7–8.7 is a straight line with a slope of about -1, the authors pointed out that a group with a pk_a at acid or alkaline pH may be involved in the formation of the enzyme substrate complex.

Chemical modification of essential amino acids

McFadden and co-workers have described the chemical modification of the ICLs from *L. usitatissimum* [32], and *Citrullus vulgaris* [61]. In studies on affinity labelling of

ICL active-site by alkylation with 3-bromopyruvate (BrP), the authors found that substrate [32, 61] or products [32] protect ICL against BrP inactivation, suggesting that the alkylating agent interacts with the enzyme active-centre. BrP was found to modify one cysteine residue per monomer which probably belongs to the glyoxylate or both glyoxylate and succinate moiety [32].

Chemical modification by diethylpyrocarbonate was studied in *L. usitatissimum* [32] and *Citrullus vulgaris* [61] enzymes whose active site was found to contain a histidine residue involved in catalysis. The authors [32] also pointed out that this finding might agree with the results of pH-optimum studies (see above).

Itaconate epoxide, an ICL active-site directed reagent, has been used to label the active site of *Citrullus vulgaris* ICL [61]. Hydroxylamine reverses the enzyme inactivation, providing evidence for the formation of an ester-linkage between itaconate epoxide and a carboxylate (aspartate or glutamate) group of ICL active-site. As proposed by the authors, this carboxylate group may be involved in the coordination of Mg^{2+} at the enzyme-active centre. The removal of the *C*-terminal amino acid (a histidine residue, see above) causes inactivation of *L. usitatissimum* ICL [32]. It is not known whether the terminal residue is the same one as that involved in the active site.

Catalytic mechanism

ICL is believed to act by acid–base catalysis through a proton transfer. The formation of a carbanion as intermediate in the catalytic mechanism appears very likely and some evidence to that effect has been reported for *Ricinus* ICL [64]. Studies by active-site modifications showed that histidine, cysteine and carboxylate residues may be involved in this mechanism (see above). The role of Mg^{2+} in catalysis is as yet not clear. Some data suggest an interaction of this ion with the substrate and/or active site (see above).

An earlier reaction scheme for ICL from the bacterium *P. indigofera* was based on data from bacterial ICL [53]. According to this scheme Mg ions play a role similar to that in class II aldolases. More recently, Eggerer and co-workers [65] have pointed out that the isocitrate cleavage by this mechanism would require the oxidation of the substrate to α-ketotricarballilate. In order for this to happen, ICL would have to contain a coenzyme (i.e. NAD) which has never been found in any of the ICLs studied to date. Schloss and Cleland [66] supposed that succinate binds as an 'aci' form to *Pseudomonas indigofera* ICL-active site. As it has been reported elsewhere [30] plant ICLs seem to have the same kinetic mechanism and similar molecular properties as compared with bacterial enzyme. Thus, prokaryotic and higher plant ICLs have probably similar catalytic mechanisms.

CONCLUSION

Many recent papers have reported the existence of multiple ICL forms which are probably related to proteolysis. Indeed, proteolytic artifacts can occur after cell rupture; however there are indications that one or more proteases selectively inactivate ICL and might therefore play an essential role in the enzyme's regulation *in vivo*. We have recently shown proteolytic activity to be tightly adsorbed to *Pinus pinea* ICL in highly purified preparations—a promising area for further investigation.

The ICL kinetic mechanism has been characterized well enough and is probably the same for all higher plant ICLs. Most likely it is also identical to that of the bacterial enzyme. Little information exists, however, about the real role of magnesium (often described as an 'essential activator') in catalysis; in particular, it is still unclear whether ICL's true substrate is the magnesium-isocitrate complex or free isocitrate. The answer to this question would provide a better insight into the catalytic mechanism of ICL.

Our understanding of the structural features of ICL is still at a primitive level. Data on even its partial amino acid sequences (i.e. active site sequences) would make possible a significant evolutionary comparison. The outline of ICL evolution would be especially meaningful since this enzyme is virtually lacking among higher animals. Little work has been published on the secondary and tertiary structure of ICL and doubts remain about both the structural and functional identity of its subunits.

The glycoprotein nature of ICL is another controversial issue whose solution would help to clarify the mechanism of ICL import into glyoxysomes. Indeed, protein glycosylation occurs only in endosperm endoplasmic reticulum, as Trelease has pointed out. Although there have been studies of the effect of various metabolites on ICL, little information is available about the enzyme's regulation *in vivo*. In our opinion, ICL amply deserve further attention and study by researchers in enzymology.

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