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Cloning, Sequence Analysis, Expression, and Inactivation of the *Corynebacterium glutamicum icd* Gene Encoding Isocitrate Dehydrogenase and Biochemical Characterization of the Enzyme

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NADP$^+$-dependent isocitrate dehydrogenase (ICD) is an important enzyme of the intermediary metabolism, as it controls the carbon flux within the citric acid cycle and supplies the cell with 2-oxoglutarate and NADPH for biosynthetic purposes. In the amino acid-producing organism *Corynebacterium glutamicum*, the specific activity of ICD was independent of the growth substrate and of the growth phase at approximately 1 U/mg, indicating that this enzyme is constitutively formed. The ICD gene, *icd*, was isolated, subcloned on a plasmid, and introduced into *C. glutamicum*. Compared with the wild type, the recombinant strains showed up to 10-fold-higher specific ICD activities. The nucleotide sequence of a 3,595-bp DNA fragment containing the *icd* gene was determined. The predicted gene product of *icd* consists of 739 amino acids (M_r = 80,091) and showed 58.5% identity with the monomeric ICD isozyme II from *Vibrio* sp. strain ABE-1 but no similarity to any known ICD of the dimeric type. Inactivation of the chromosomal *icd* gene led to glutamate auxotrophy and to the absence of any detectable ICD activity, suggesting that only a single ICD is present in *C. glutamicum*. From an *icd*-overexpressing *C. glutamicum* strain, ICD was purified and biochemically characterized. The native ICD was found to be a monomer; to be specific for NADP$^+$; to be weakly inhibited by oxaloacetate, 2-oxoglutarate, and citrate; and to be severely inhibited by oxaloacetate plus glyoxylate. The data indicate that ICD from *C. glutamicum* is structurally similar to ICDs from bacteria of the genera *Vibrio*, *Rhodomicrobium*, and *Azotobacter* but different from all other known procaryotic and eucaryotic ICDs.

NADP$^+$-dependent isocitrate dehydrogenase (ICD; EC 1.1.1.42) is a key enzyme of the citric acid cycle, and it catalyzes the oxidative decarboxylation of D-isocitrate to form 2-oxoglutarate, CO_2, and NADPH. 2-Oxoglutarate can be further oxidized within the cycle or reductively aminated to glutamate, and NADPH can be used for reductive biosynthetic reactions. Thus, the ICD supplies the cell with a key intermediate of the energy metabolism as well as with precursors and reducing power for anabolic pathways, e.g., amino acid biosynthesis. In aerobic organisms able to grow on acetate as the sole carbon source, ICD plays a further role, namely, in cocontrolling the carbon flux at the branch point between the citric acid cycle and the glyoxylate cycle which, during growth on acetate, is essential as an anaplerotic pathway (29). Isocitrate lyase, the first enzyme of the glyoxylate cycle, competes with ICD for their common substrate isocitrate. The carbon flux to either the citric acid cycle or the glyoxylate cycle is controlled by changing the activity of either one of the two enzymes and/or by changing their affinities towards isocitrate (30, 38, 55). In *Escherichia coli*, isocitrate lyase is formed only during growth on acetate (29) and ICD is regulated via reversible phosphorylation (14) by a bifunctional ICD kinase/phosphatase (reviewed in references 30 and 38). When acetate is the sole carbon source, the ICD enzyme is phosphorylated and thus inactivated, thereby decreasing the carbon flow through the citric acid cycle and allowing the operation of the glyoxylate bypass (30, 31, 38, 57).

Because of their roles in energy production and intermediary metabolism, the ICDs of a variety of organisms have been purified and extensively studied with respect to structural, kinetic, and regulatory characteristics (5). In most procaryotes, ICD is a dimeric enzyme consisting of identical subunits with molecular weights of between 40,000 and 57,000 (5, 35). However, *Vibrio parahaemolyticus*, *Rhodomicrobium vannielii*, and *Azotobacter vinelandii* possess monomeric ICDs with molecular weights of about 80,000 (6, 13, 32). Both the dimeric and the monomeric type of ICD have been found in *Vibrio* sp. strain ABE-1 (24). Despite the differences in subunit size and number, all bacterial ICDs studied so far are similar with respect to specific activity, requirement of Mn^{2+} or Mg^{2+} for activity, inhibition by oxaloacetate plus glyoxylate, and their high affinities towards isocitrate (5).

Besides the biochemical characterization of numerous ICDs, genes coding for this enzyme have been cloned and sequenced from some procaryotes, i.e., from *E. coli* (52), *Thermus thermophilus* (34), *Anabaena* sp. strain PCC7120 (35), and *Bacillus subtilis* (26), as well as from some eucaryotes (7, 17, 18, 21, 54). Comparison of the deduced amino acid sequences revealed conserved regions in all ICDs (7, 35). Recently, the genes coding for both the dimeric ICD-I and the monomeric ICD-II from *Vibrio* sp. strain ABE-1 have also been sequenced, and it was revealed that the deduced primary structure of ICD-II has no similarity to any other ICD sequence (25). This result suggests a clear distinction between monomeric and dimeric ICDs; however, characterization of other genes coding for monomeric ICDs is necessary to sustain this hypothesis.

The gram-positive bacterium *Corynebacterium glutamicum* is widely used for the industrial production of amino acids, e.g., L-glutamate and L-lysine (27). This organism grows aerobically on glucose and on acetate, and both carbon sources can also

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Source[b] or reference
Strains		
E. coli EB106	*icd-11 dadR1 trpA62 trpE61 tna-5* lambda$^-$	1
E. coli DH5	*supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	16
E. coli S17-1	*thi-1 endAR1 hsdR17* (r^- m^+) *supE44 pro*	50
C. glutamicum WT	WT strain ATCC 13032	ATCC
C. glutamicum subsp. *flavum*	ATCC 14067, formerly *Brevibacterium flavum*	ATCC
C. glutamicum subsp. *lactofermentum*	ATCC 13869, formerly *Brevibacterium lactofermentum*	ATCC
C. glutamicum DOR	ATCC 13032 *icd*::pSUP301::icd_{int}	This work
Cosmids or plasmids		
pHC79-based gene library	*C. glutamicum* chromosomal DNA cloned in cosmid pHC79	4
pHC-icd	Recombinant pHC79 cosmid able to complement *E. coli* EB106	This work
pEK0	Km^r, *ori* of ColE1, *ori* of pBL1	10
pEK-icdE1 and -E2	pEK0 containing a 5.6-kb *Eco*RI fragment from pHC-icd	This work
pEK-icdES1	pEK0 containing a 3.6-kb *Eco*RI-*Sal*I fragment from pEK-icdE1	This work
pEK1.9gdh	pEK0 carrying the *C. glutamicum* WT *gdh* gene	4
pEKgdh-icd	pEK1.9gdh containing a 3.6-kb *Eco*RI-*Sal*I fragment from pEK-icdES	This work
pUC18	Ap^r, *ori* of ColE1	56
pSUP301	Mobilizable vector, Km^r *oriT*	50
pGEM-4Z	Transcription vector carrying the T7 and SP6 promoters, Ap^r	Promega Inc.

[a] Km^r, kanamycin resistance; Ap^r, ampicillin resistance.
[b] ATCC, American Type Culture Collection.

serve as substrates for the production of amino acids (28). Accordingly, both the citric acid cycle and the glyoxylate cycle are present in *C. glutamicum* (27, 39). In an initial attempt to investigate the carbon flux control between these two cycles, we started genetic and biochemical studies on isocitrate lyase and ICD from this organism. Recently, we characterized the isocitrate lyase and the corresponding gene and showed that formation of the enzyme is tightly regulated by the carbon source of the medium and that the enzyme activity is inhibited by intermediates of the central metabolism (41). In this communication, we report the cloning and sequence analysis of the *C. glutamicum* ICD gene, its expression, and its inactivation within the chromosome. Furthermore, we present biophysical and biochemical data about the purified ICD enzyme.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids, their relevant characteristics, and their sources or references are given in Table 1. M9 medium (42) containing 0.02 mM thiamine was used as a minimal medium (MM) for *E. coli*; in the case of *E. coli* EB106, L-glutamate and L-tryptophan were added at 3 and 1 mM, respectively. The MM used for *C. glutamicum* has been described previously (11), and it contained 4% glucose, 2% lactate, 2% acetate, or 1% glucose plus 1% acetate. Luria-Bertani (LB) medium (42) was used as a complex medium for all organisms. When appropriate, ampicillin (100 μg/ml) or kanamycin (50 μg/ml) was added to the medium. *E. coli* was grown aerobically at 37°C, and *C. glutamicum* was grown at 30°C.

DNA preparation and transformation. Plasmids from *E. coli* were isolated by the method of Birnboim (3). Plasmids and chromosomal DNA from *C. glutamicum* were obtained as described elsewhere (12). *E. coli* was transformed by electroporation or by the $CaCl_2$ method (42); *C. glutamicum* was transformed by electroporation (33).

DNA manipulations. Restriction enzymes, T4 DNA ligase, Klenow polymerase, proteinase K, DNase I, RNase A, RNasin, and T7 RNA polymerase were obtained from Boehringer (Mannheim, Germany) or from Promega (Madison, Wis.) and used as instructed by the manufacturers.

DNA hybridization experiments were performed as previously described (41). The 3.6-kb *Eco*RI-*Sal*I fragment isolated from plasmid pEK-icdE1 was labeled with digoxigenin-dUTP and used as a probe. Labeling, hybridization, washing, and detection were performed with the Non-radioactive DNA Labeling and Detection Kit from Boehringer.

For sequencing, the 2.02-kb *Eco*RI-*Bam*HII fragment and the 2.05-kb *Hpa*I-*Sal*I fragment from pEK-icdE1 were blunt ended and ligated into plasmid pUC18. From the resulting plasmids progressive unidirectional deletions of the inserted DNA were created with the Erase-a-base kit from Promega. Appropriate subclones were sequenced by the dideoxy chain termination method (43), using the AutoRead sequencing kit from Pharmacia (Freiburg, Germany) with subsequent electrophoretic analysis with an A.L.F. DNA sequencer from Pharmacia. Sequence data were compiled and analyzed with the HUSAR program package (European Molecular Biology Laboratory, Heidelberg, Germany).

RNA hybridization. Total RNA from *C. glutamicum* was isolated and purified as described previously (4), except that the phenol extraction temperature was 65°C. For hybridization, 10 μg of this RNA was separated on an agarose gel containing 17% formaldehyde and transferred onto a nylon membrane (4). An *icd* antisense RNA probe was prepared by ligating the 2.02-kb *Eco*RI-*Bam*HI fragment (see Fig. 1) into plasmid pGEM-4Z, linearizing the resulting plasmid with *Sma*I, and synthesizing digoxigenin-dUTP-labeled RNA, using T7 RNA polymerase and the RNA Labeling Kit from Boehringer. Hybridization (at 46°C, in the presence of 50% formamide), washing, and detection were performed with the Nucleic Acid Detection Kit from Boehringer. The size marker was the 0.36- to 9.5-kb RNA ladder from Promega.

Gene disruption. The chromosomal *icd* gene was disrupted by a method described by Schwarzer and Pühler (45). An *icd*-internal 0.33-kb *Xba*I-*Hpa*I fragment from plasmid pEK-icdES1 was blunt ended and ligated into the *Sca*I site of the mobilizable *E. coli* vector pSUP301, which is nonreplicative in *C. glutamicum*. The resulting plasmid, pSUP-icd_{int}, was introduced into *C. glutamicum* via conjugation from *E. coli* S17-1 by the method of Schäfer et al. (44); the transconjugants were selected on brain heart infusion agar plates containing kanamycin (15 μg/ml) and nalidixic acid (50 μg/ml). Southern blot analysis was performed to prove the integration of pSUP-icd_{int} at the chromosomal *icd* locus of one of the transconjugants, *C. glutamicum* DOR. *Eco*RI-*Sal*I-restricted chromosomal DNA from this strain was hybridized to the labeled 3.6-kb *Eco*RI-*Sal*I fragment from pEK-icdE1, resulting in a signal at 9.3 kb. The size of the chromosomal *Eco*RI-*Sal*I fragment in strain DOR was expected to be 9.3 kb, i.e., 3.6 kb of the original fragment plus 5.7 kb of pSUP-icd_{int} DNA.

Glutamate fermentation. Glutamate secretion was analyzed by using short-term fermentations as described elsewhere in detail (19). *C. glutamicum* was grown under biotin limitation (1 μg/liter) in MM with glucose, harvested in the early production phase, and resuspended in the same medium. The cell suspensions with an optical density at 600 nm of ~5 were incubated at 30°C and stirred at 650 rpm.

Enzyme assay. To determine ICD activity, cells were grown in 60 ml of medium in baffled 500-ml Erlenmeyer flasks, washed twice in 20 ml of 100 mM potassium phosphate buffer (pH 7), and resuspended in 1 ml of the same buffer. Cells were disrupted as previously described (41), and after centrifugation for 30 min at 4°C and 13,000 × *g*, the supernatant was used as a cell extract. The protein concentration was determined by the biuret method (15), using bovine serum albumin as the standard. The ICD was assayed photometrically as described by Nachlas et al. (36) at 30°C in 1 ml of a solution of 100 mM triethanolamine (TEA) buffer (pH 7.6), 0.8 mM $MnSO_4$, 0.5 mM NADP, and 0.8 mM D$_S$-*threo*-isocitrate as a substrate. For determination of the pH optimum, 100 mM 2-morpholineethanesulfonic acid (MES) buffers with pHs of 6.0 to 7.2, 100 mM TEA buffers with pHs of 6.9 to 8.2, and Tris-HCl buffers with pHs of 7.8 to 9.0 were used. The increase of NADPH was monitored at 340 nm. One unit of activity is defined as 1 μmol of NADPH formed per min.

Purification of ICD. Cell extracts for purification of ICD were prepared as described above except that the cells were resuspended in 2 ml of buffer A (50 mM MES buffer [pH 6.0] containing 5 mM $MnSO_4$ and 5 mM dithiothreitol). All purification steps were carried out at 4°C. After the addition of 5 U of DNase per ml and 20 μg of RNase per ml, the cell extract was subjected for 2 h to ultracentrifugation at 145,000 × *g*. The supernatant was then loaded onto a MonoQ HR5/5 column, and after a washing with buffer A, the enzyme was eluted with a linear 0.15 to 0.4 M NaCl gradient in the same buffer and with a flow rate of 0.7 ml/min. From the start of the gradient, fractions of 1 ml were collected and tested for ICD activity. The ICD enzyme eluted at 0.26 M NaCl.

SDS-PAGE and gel filtration. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as previously described (41). Gel filtration was performed at 4°C by fast protein liquid chromatography, using a Superose 12 HR10/30 column (Pharmacia) equilibrated with 100 mM TEA buffer (pH 7.5) containing 5 mM dithiothreitol, 5 mM $MgSO_4$, and 1 mM D,L-isocitrate. Before cell extract was applied to the column, the buffer of the extract was replaced by the equilibration buffer, by using a PD-10 column (Pharmacia). The flow rate during gel filtration was 0.3 ml/min. Protein standards were ferritin (M_r = 440,000), catalase (M_r = 232,000), β-amylase (M_r = 200,000), alcohol dehydrogenase (M_r = 150,000), glucose-6-phosphate dehydrogenase (M_r = 110,000), albumin (M_r = 67,000), ovalbumin (M_r = 43,000), and chymotrypsinogen (M_r = 25,000).

Nucleotide sequence accession number. The nucleotide sequence reported in this work has been assigned GenBank accession number X71489.

RESULTS

ICD activity in *C. glutamicum*. The $NADP^+$-dependent ICD activity in cell extracts of *C. glutamicum* wild type (WT) was assayed after growth on LB medium minus and plus glucose and on MM containing glucose, lactate, acetate, or a mixture of glucose plus acetate. The specific ICD activity was in all cases between 0.9 and 1.1 U/mg of protein and thus independent of the carbon source in the growth medium. The specific activity was also identical when the cells were harvested at the early, mid-, or late exponential growth phase or at the stationary growth phase. NAD^+-linked ICD activity was not found under any conditions. These results indicate that *C. glutamicum* possesses a highly active and constitutively formed $NADP^+$-specific ICD and suggest the absence of an NAD^+-dependent ICD (EC 1.1.1.41) or of an enzyme with dual coenzyme specificity as found, e.g., in *R. vannielii* (32).

Isolation of the *icd* gene from *C. glutamicum*. The *C. glutamicum icd* gene encoding ICD was isolated by heterologous complementation of the *E. coli* mutant EB106. Because of its ICD deficiency, *E. coli* EB106 requires glutamate during growth on glucose MM, and plasmids or cosmids carrying a functional *icd* gene can be identified by complementation of this auxotrophy. A cosmid gene library consisting of *Sau*3A-generated chromosomal *C. glutamicum* WT DNA fragments ligated into the *Bam*HI site of the cosmid vector pHC79 (4) was used to transform *E. coli* EB106. After selection on LB agar plates containing ampicillin, approximately 500 transformants were screened for complementation of the *icd* marker (glutamate auxotrophy) by being replica plated onto glucose MM. One clone which grew on glucose in the absence of glutamate was obtained. After isolation of the cosmid pHC-icd and retransformation of *E. coli* EB106, all transformants again grew on glucose MM, suggesting that the cosmid carried the *C. glutamicum icd* gene.

In order to subclone the *icd* gene, cosmid pHC-icd was restricted with *Eco*RI, and the resulting fragments were ligated into the *Eco*RI site of the *E. coli-C. glutamicum* shuttle vector pEK0. The ligation mixture was used to transform *E. coli* EB106, and kanamycin-resistant transformants were again screened for complementation. By this procedure, several clones able to grow on glucose MM without glutamate were obtained. The plasmids isolated from these clones all contained a 5.6-kb *Eco*RI fragment (Fig. 1) inserted in either one or the other direction and were designated pEK-icdE1 and pEK-icdE2, respectively. For further confinement of the *icd* gene, smaller fragments were isolated out of plasmid pEK-icdE1 and tested for complementation ability. By this procedure, we obtained plasmid pEK-icdES1, which contains a 3.6-kb *Eco*RI-*Sal*I fragment (Fig. 1) and is able to complement the *icd* mutation of *E. coli* EB106.

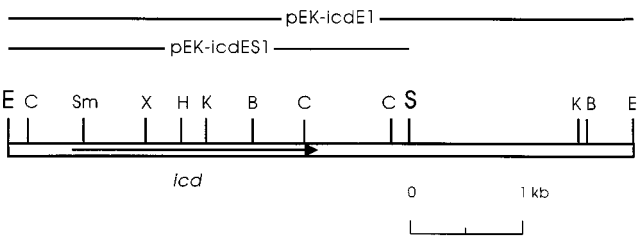

FIG. 1. Restriction map of the *C. glutamicum* chromosomal 5.6-kb DNA fragment containing the *icd* gene. The bars above the map indicate those regions which are present in plasmids pEK-icdE1 (and pEK-icdE2) and pEK-icdES1. The 3.6-kb fragment sequenced is indicated by boldface letters. The arrow represents the predicted *icd* coding region. Abbreviations: B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hpa*I; K, *Kpn*I; S, *Sal*I; Sm, *Sma*I; X, *Xba*I.

To confirm the origin of the 3.6-kb *Eco*RI-*Sal*I fragment, genomic DNA from *C. glutamicum* was analyzed by Southern hybridization. For this purpose, the *Eco*RI-*Sal*I fragment was isolated from pEK-icdE1 and labeled with digoxigenin-dUTP. Hybridization of this probe to *Eco*RI-*Sal*I-restricted and size-fractionated chromosomal DNA from *C. glutamicum* WT resulted in a signal at 3.6 kb. This result demonstrates that the cloned fragment originated from *C. glutamicum* and that it corresponds to a genomic fragment with no detectable structural alteration. The labeled probe was also hybridized to *Eco*RI-*Sal*I-restricted chromosomal DNAs from *C. glutamicum* subsp. *flavum* and subsp. *lactofermentum*. These hybridizations also resulted in specific signals at 3.6 kb, indicating that a DNA fragment identical to the ICD complementing fragment from *C. glutamicum* WT is present in the two subspecies.

Expression of the cloned *icd* gene. In order to assess the *icd*-complementing fragments for expression of a functional *icd* gene, plasmids pEK-icdE1, pEK-icdE2, and pEK-icdES1 were transformed into *C. glutamicum* WT, and the specific ICD activities in cell extracts of the resulting strains were determined. As shown in Table 2, *C. glutamicum* strains harboring pEK-icdE1 and pEK-icdES1 and that harboring pEK-icdE2 displayed about 10- and about 6-fold-higher activities of ICD, respectively, than did the host strain or its derivative carrying the cloning vector pEK0. This is in the same range as previously observed with other enzymes when the corresponding

TABLE 2. Specific activities of ICD in cell extracts of different *C. glutamicum* strains

C. glutamicum strain	Sp act (U/mg of protein)[a]
WT	1.0 ± 0.15
WT(pEK0)	0.9 ± 0.10
WT(pEK-icdE1)	10.7 ± 0.8
WT(pEK-icdE2)	6.3 ± 0.3
WT(pEK-icdES1)	9.9 ± 0.8
DOR	<0.001

[a] Mean values ± standard deviations were obtained from at least three independent cultivations by two determinations per experiment. Specific activities were obtained by using cells grown on LB medium. When the cells were grown on MM plus glucose or plus acetate, the specific activities were essentially the same.

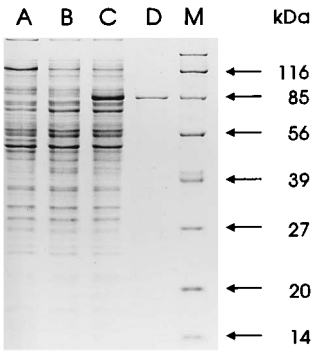

FIG. 2. SDS-PAGE of cell extracts from *C. glutamicum* DOR (lane A), *C. glutamicum* WT(pEK0) (lane B), *C. glutamicum* WT(pEK-icdES1) (lane C), and purified ICD (lane D). Lane M, molecular mass standards (i.e., β-galactosidase [116 kDa], fructose-6-phosphate kinase [85 kDa], glutamate dehydrogenase [56 kDa], aldolase [39 kDa], triosephosphate isomerase [27 kDa], trypsin inhibitor [20 kDa], and lysozyme [14 kDa]).

genes were cloned on pEK0 and introduced into *C. glutamicum* (4, 12). The slightly higher ICD activities of *C. glutamicum* WT(pEK-icdE1) and WT(pEK-icdES1) compared with that of *C. glutamicum* WT(pEK-icdE2) might be due to additional transcription by readthrough from the *lac* promoter located in front of the multiple cloning site of plasmid pEK0. However, these results show that the cloned fragments contain a functional *icd* gene which is expressed in *C. glutamicum.*

Cell extract of *C. glutamicum* WT(pEK-icdES1) was also analyzed by SDS-PAGE. A dominant protein band was observed at a molecular weight of approximately 85,000 (Fig. 2, lane C). The corresponding protein band was much weaker in cell extracts of *C. glutamicum* WT(pEK0) (Fig. 2, lane B), suggesting that this band represents the ICD expressed from the cloned fragment. Since a molecular weight of 80,000 is typical of monomeric ICDs (5), this result indicated for the first time that *C. glutamicum* may possess an ICD of the monomeric type.

C. glutamicum WT and the *icd*-overexpressing strains grew equally well on MM containing glucose or lactate (doubling time, 100 min) but showed different growth rates when acetate was used as the sole carbon source. Under these conditions, the doubling time for *C. glutamicum* WT(pEK-icdE1) and WT (pEK-icdES1) was approximately 250 min compared with 160 min for the WT strain.

Effect of *icd* overexpression on glutamate formation. *C. glutamicum* is well known to secrete glutamate under certain conditions (27, 28). Since ICD is responsible for supplying 2-oxoglutarate, the direct precursor of glutamate, it was interesting to study the influence of *icd* overexpression on this organism's ability to secrete glutamate. For this purpose, standard glutamate fermentations (19) were performed with *C. glutamicum* WT and WT(pEK-icdES1). Both strains secreted glutamate at identical rates of about 19 μmol min^{-1} g (dry weight)$^{-1}$, showing that the capacity of *C. glutamicum* to secrete glutamate cannot be enhanced simply by elevating the ICD enzyme level. This result and the findings that the ICD in *C. glutamicum* is highly active and constitutively present indicate that a factor other than ICD is rate limiting for glutamate production by this organism.

The *icd* gene was also overexpressed together with the glutamate dehydrogenase (GDH) gene (*gdh*), which had been previously isolated and characterized (4). GDH catalyzes the reductive amination of 2-oxoglutarate to glutamate with concomitant oxidation of NADPH and has been discussed as being tightly linked to the $NADP^+$-dependent ICD in glutamate-forming corynebacteria (27). For combined overexpression of *icd* and *gdh*, the 3.6-kb *Eco*RI-*Sal*I fragment of pEK-icdES1 was ligated into plasmid pEK1.9gdh, and the resulting plasmid pEKgdh-icd was transformed into *C. glutamicum* WT. The recombinant strain showed an eightfold-higher specific activity of ICD (7.9 U/mg) and a sixfold-higher specific activity of GDH (8.4 U/mg) compared with those of the parental WT strain (ICD, 0.96 U/mg, and GDH, 1.44 U/mg). However, in standard glutamate fermentations, the *icd*- and *gdh*-overexpressing *C. glutamicum* strain secreted glutamate at the same rate (19 μmol min^{-1} g^{-1}) as the WT strain. Thus, the potential of *C. glutamicum* to secrete glutamate could also not be enhanced by increasing the level of both ICD and GDH.

Nucleotide sequence of the *icd* gene. The nucleotide sequence of the 3,595-bp *Eco*RI-*Sal*I fragment was determined from both strands by the dideoxy chain termination method and is shown in Fig. 3. Computer analysis revealed three open reading frames (ORFs) extending from the beginning of the fragment to bp 483 (ORF1), from bp 567 to 2783 (ORF2), and from bp 2863 to 3567 (ORF3). ORF1 and ORF3 exhibited a codon usage untypical of corynebacterial genes (9), and database searches with the nucleotide sequences and the deduced polypeptides of these two ORFs revealed no similarity to sequences stored in the GenBank and SwissProt databases. In contrast, ORF2 exhibited a codon usage matching perfectly to that of highly expressed *C. glutamicum* genes (9), and the amino acid sequence encoded by this ORF revealed significant identity to the monomeric ICD of *Vibrio* sp. strain ABE-1 (see below). These results indicate that ORF2 represents the *icd* gene from *C. glutamicum*. The *icd* gene is preceded by a typical ribosomal binding site (AAGGAG) and followed by a structure resembling rho-independent transcription terminators (Fig. 3). According to the rules of Tinoco et al. (53), the mRNA hairpin loop predicted from this sequence has a ΔG value of −35.6 kcal (ca. −148.9 kJ)/mol at 25°C. The predicted *icd* gene product consists of 739 amino acids with a molecular weight of 80,091, which is in agreement with the size of the intense protein band observed in the cell extracts of *C. glutamicum* WT(pEK-icdES1) (Fig. 2, lane C) and with the molecular weight typical of monomeric ICDs ($M_r \approx 80{,}000$) (5).

Analysis of the deduced ICD amino acid sequence from *C. glutamicum*. GenBank and SwissProt database searches with the deduced amino acid sequence of the *C. glutamicum* ICD revealed close matches to the complete amino acid sequence of the monomeric ICD-II from *Vibrio* sp. strain ABE-1 (25) and to the N-terminal sequences of the monomeric ICDs from *V. parahaemolyticus* (13) and *R. vannielii* (32) (Fig. 4). In an alignment, the *C. glutamicum* enzyme shows 58.5% identity to the *Vibrio* sp. strain ABE-1 enzyme, and considering conservative exchanges, similarity is as high as 70.1%. From the *V. parahaemolyticus* and *R. vannielii* ICDs, only the N-terminal amino acid sequences (up to amino acids 28 and 39, respectively) have been determined. However, they show 50% (*V. parahaemolyticus*) and 53.8% (*R. vannielii*) identity to the N-terminal part of the *C. glutamicum* ICD. No similarity could be detected between the amino acid sequence of the *C. glutamicum* enzyme and any known sequence of dimeric ICDs, e.g., from *E. coli* (52), *T. thermophilus* (34), *Anabaena* sp. strain PCC7120 (35), *B. subtilis* (26), *Saccharomyces cerevisiae* (7, 18), soybean (54), and bovine or porcine mitochondria (17, 21), although all these share between 30 and 66% identity with each other (7, 26, 35). These results indicate that the *C. glutamicum*

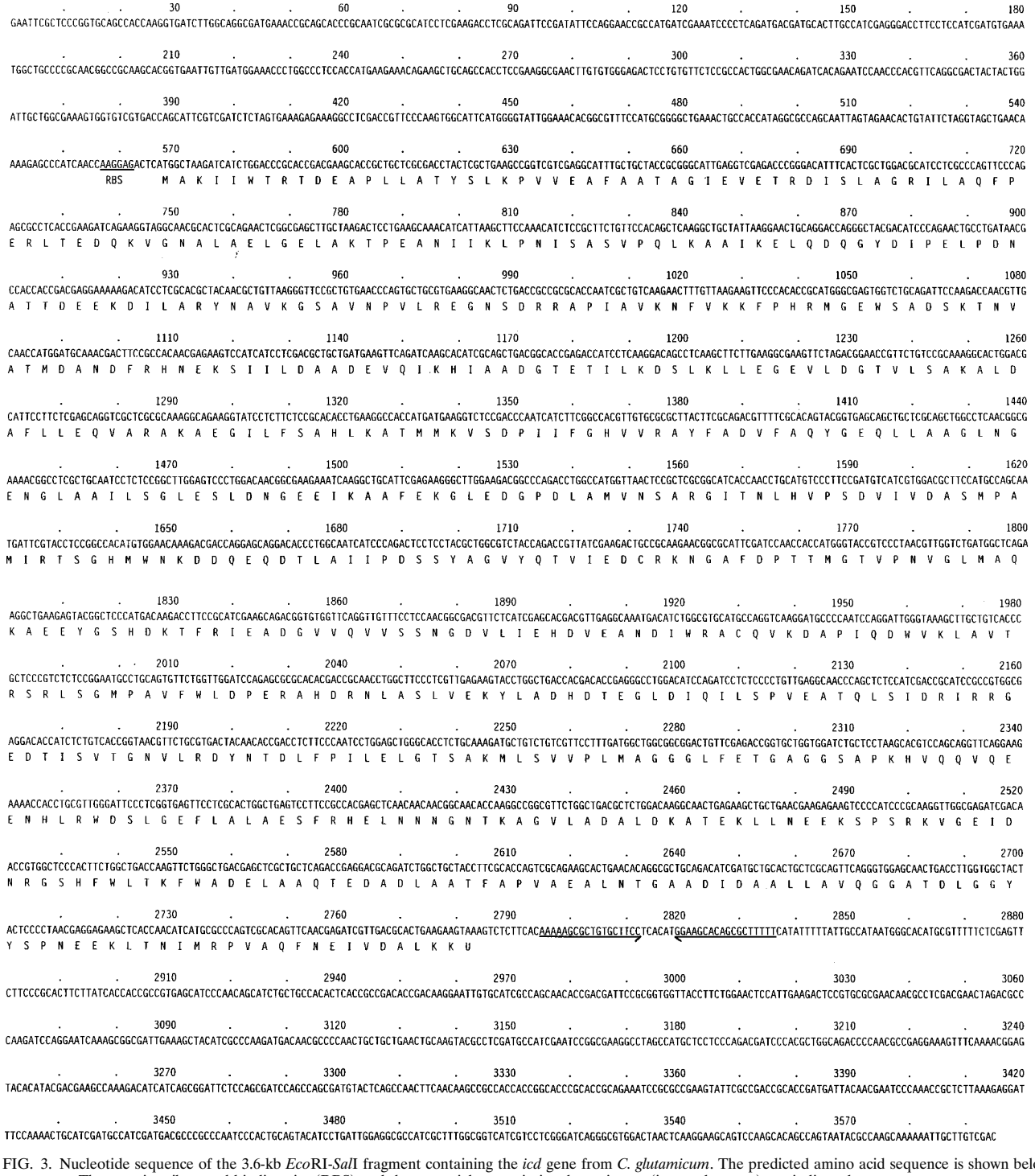

FIG. 3. Nucleotide sequence of the 3.6-kb *Eco*RI-*Sal*I fragment containing the *icd* gene from *C. glutamicum*. The predicted amino acid sequence is shown below the sequence. The putative ribosomal binding site (RBS) and the potential transcriptional terminator (inverted arrows) are indicated.

ICD is highly homologous to the hitherto known monomeric ICDs and not related to dimeric ICDs.

Northern (RNA) analysis of the *icd* gene. RNA hybridization experiments were performed to identify the *icd* mRNA transcript(s). Total RNA was isolated from *C. glutamicum* WT, size fractionated, blotted onto a nylon membrane, and hybridized to an *icd*-specific digoxigenin-labeled antisense RNA probe. As shown in Fig. 5, hybridization to the *icd* probe resulted in a signal at 2.4 kb. This size corresponds approximately to that of the structural *icd* gene (2,217 bp) and indicates that the *C. glutamicum icd* gene is monocistronic. Thus, the transcriptional organization is similar to that of the monomeric ICD gene (*icdII*) from *Vibrio* sp. strain ABE-1 (25).

Inactivation of the chromosomal *icd* gene in *C. glutamicum*. To study whether *C. glutamicum* requires the *icd* gene for growth, the chromosomal copy of this gene was disrupted in *C.*

```
C. glu.  M----AKIIW TRTDEAPLLA TYSLKPVVEA FAATAGIEVE TRDISLAGRI LAQFPERLTE DQKVGNALAE LGELAKTPEA NIIKLPNISA SVPQLKAAIK   96
V. sp.   MSTDNSKIIY TITDEAPALA TYSLLPIEQA YTASSGINVE TRDISLAGRI LANFPKYLTK EQRIDDALAE LGELAQTPEA NIIKLPNISA SIPQLEAVIK  100
V. par.   STEKPTIIY TITDEAPALA TYSNLPIIQ
R. van.     NEAPTIVW TRTDESPALA SYSLLPIVQA FTETAGVSVV Q

C. glu.  ELQDQGYDIP ELPDNATTDE EKDILARYNA VKGSAVNPVL REGNSDRRAP IAVKNFVKKF PHRMGEWSAD SKTNVATMDA NDFRHNEKSI ILDAADEVQI  196
V. sp.   ELQAKGYDLP HYPAEPQNEA EESIKLTYAK ILGSAVNPVL REGNSDRRAP ASVKQYARNN PHSMGAPSKE SKSHVAHMAS GDFYGSEKSV TIDGATSVNI  200

C. glu.  KHIAADGTET ILKDSLKLLE GEVLDGTVLS AKALDAFLLE QVARAKAEGI LFSAHLKATM MKVSDPIIFG HVVRAYFADV F---AQYGEQ LLAAGLNGEN  293
V. sp.   EFVAKNGDVT LLKSKLPLLD KEIIDASVMS KSALVEFFET EINKAKEEDV LLSLHLKATM MKVSDPVMFG HAVRVFYKDV FAKHAATFEQ LGVDADNGIG  300
A. vin.                        G KSALRKFIAA QIEDAKXGXV LLXVXLKATM M

C. glu.  GLAAILSGLE SLDNGEEIKA AFEKGLEDGP DLAMVNSARG ITNLHVPSDV IVDASMPAMI RTSGHMWNKD DQEQDTLAII PDSSYAGVYQ TVIEDCRKNG  393
V. sp.   DVYAKIARL- PAAQKEEIEA DLQAVYATRP EMAMVDSDKG ITNLHVPSDV IIDASMPAAL RASGMMWGPD GKQKDTKFMI PDRNYAGVFS AVVDFCRENG  399

C. glu.  AFDPTTMGTV PNVGLMAQKA EEYGSHDKTF RIEADGVVQV VSSNGDVLIE HDVEANDIWR ACQVKDAPIQ DWVKLAVTRS RLSGMPAVFW LDPERAHDRN  493
V. sp.   AFNPATMGTV PNVGLMAQKA EEYGSHDKTF TMKAAGTVRV VNSQGERLIE QEVAQGDIYR MCQVKDAPIQ DWVKLAVTRA RATGTPTVFW LDENRGHDEQ  499

C. glu.  LASLVEKYLA DHDTEGLDIQ ILSPVEATQL SIDRIRRGED TISVTGNVLR DYNTDLFPIL ELGTSAKMLS VVPLMAGGGL FETGAGGSAP KHVQQVQEEN  593
V. sp.   MIKKVNTYLA DHDTTGLDIQ ILEPVKACES TLARVAKGED AISVTGNVLR DYLTDLFPIL ELGTSAKMLS IVPLMNGGGL VETGAGGSAP KHVQQFEKEN  599

C. glu.  HLRWDSLGEF LALAESFRHE LNNNGNTKAG VLADALDKAT EKLLNEEKSP SRKVGEIDNR GSHFWLTKFW ADELAAQTED ADLAATFAPV AEALNTGAAD  693
V. sp.   HLRWDSLGEF LALAASLEHV AVTTGNARAQ ILADTLDAAT GKFLDTNKSP SRKVGELDNR VVTSIL-QCI GRNVAAQTTD TELQASFSSV AQALTKQEEK  698

C. glu.  IDAALLAVQG GATDLGGYYS PNEEKL-TNI MRPVAQFNEI VDALKK*   738
V. sp.   IVAELNAAQG PAIDLNGYY- FADTKLAEKA MRPSETFNTI LSAL-L*   742
```

FIG. 4. Alignment of the predicted amino acid sequence of the *C. glutamicum* (*C. glu.*) ICD with the sequence of ICD-II from *Vibrio* sp. strain ABE-1 (*V.* sp.) (25), with the N-terminal sequences of ICD from *V. parahaemolyticus* (*V. par.*) (13) and *R. vannielii* (*R. van.*) (32), and with an ICD-internal oligopeptide from *A. vinelandii* (*A. vin.*) (8). Symbols: ■, identical residues in all ICDs investigated; ■, identical residues in the ICDs of *C. glutamicum* and *Vibrio* sp. strain ABE-1.

glutamicum WT. The resulting strain, *C. glutamicum* DOR, was then tested for ICD activity and for growth on different media. *C. glutamicum* DOR displayed no detectable ICD activity (Table 2), and as shown by SDS-PAGE, the 85-kDa protein was absent in cell extracts of this strain (Fig. 2, lane A). In accordance with these findings, *C. glutamicum* DOR was unable to grow on MM containing glucose unless supplemented with 5 mM glutamate, whereas the growth on LB medium was slower than that of the parental strain only (doubling times of 150 and 100 min, respectively). These results show that the *icd* gene in *C. glutamicum* DOR is inactivated and that *C. glutamicum* requires a functional *icd* gene for growth on MM with glucose.

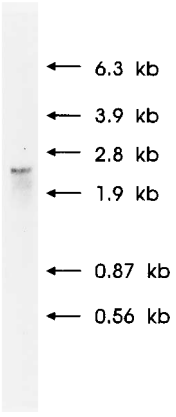

FIG. 5. Northern hybridization analysis of the *C. glutamicum icd* gene. Total RNA from *C. glutamicum* was size fractionated and probed with a digoxigenin-labeled *icd*-specific antisense RNA probe. RNA size standards are shown on the right.

Furthermore, these results strongly suggest that *C. glutamicum* WT possesses only one ICD enzyme. In most procaryotes only one ICD is present (5); however, two distinct ICD isozymes have been found in *Acinetobacter calcoaceticus* and in *Vibrio* sp. strain ABE-1 (24, 47), and eucaryotic organisms possess in general two or even more ICD isozymes (5).

Purification and characterization of ICD. In order to obtain information about biophysical and biochemical characteristics of the *C. glutamicum* ICD, the enzyme was purified from the *icd*-overexpressing strain *C. glutamicum* WT(pEK-icdES1) and analyzed. For purification, the cell extract was subjected to ultracentrifugation, and subsequently, the supernatant was chromatographed on a MonoQ anion-exchange column as described in Materials and Methods. A representative purification scheme is given in Table 3, and the result of SDS-PAGE of the purified enzyme after the last step is shown in Fig. 2 (lane D). From the purification factor of 11.77, it can be calculated that ICD represents about 8.5% of the cytoplasmic protein fraction of *C. glutamicum* WT(pEK-icdES1). This value agrees well with the relative amount of ICD that can be seen after SDS-PAGE of cell extracts from this strain (Fig. 2, lane C). The molecular weight of the purified ICD monomer determined by SDS-PAGE was 85,000, which corresponds to the molecular weight predicted from the nucleotide sequence of the *icd* gene (80,091 [see above]). By gel filtration experiments, the native molecular weight was determined to be 85,000 ± 10,000. These results show that the native ICD from *C. glutamicum* is in fact a monomeric enzyme, as was expected from the amino acid sequence comparison studies (see above).

The activity of purified ICD was stable for several days when ICD was stored at 4°C. Significant loss (70%) of activity occurred when the enzyme was frozen (−20°C) and thawed. However, in cell extracts the ICD activity was unaffected by

TABLE 3. Purification of ICD from cell extracts of *C. glutamicum* WT(pEK-icdES1)

Fraction	Vol (ml)	Total amt (mg) of protein	Total activity (U)	Sp act (U/mg)	Recovery of activity (%)	Purification (fold)
Cell extract	2.5	6.75	64.8	9.6	100	1.00
Ultracentrifugation	3.0	3.36	61.8	18.4	95	1.92
MonoQ, pH 6.0	3.0	0.36	40.5	112.5	62	11.77

repeated freezing and thawing. The activity of the enzyme was maximal over a broad pH range of 7.5 to 8.1 with 100 mM TEA buffer and decreased sharply at pH values below 6.5. The ICD showed an absolute requirement for divalent cations, which is a common feature of all NADP-dependent ICDs investigated so far (5). Mn^{2+} at 0.8 mM was found to be the most effective cation; Mg^{2+} could partially replace Mn^{2+}, resulting in approximately 40% of the maximal activity. The purified enzyme was specific for $NADP^+$ and showed no activity with NAD^+. The K_m values determined for D,L-isocitrate, D$_S$-*threo*-isocitrate, and $NADP^+$ are 23, 12, and 24 μM, respectively, and thus they are in the same range as those reported for monomeric and dimeric ICDs from other organisms (3 to 77 μM for isocitrate and 2.5 to 54 μM for $NADP^+$) (5, 32, 35).

To analyze whether ICD is regulated at the level of enzyme activity, its inhibition by different effectors was investigated. The enzyme was found to be completely inhibited by a mixture of 1 mM oxaloacetate plus 1 mM glyoxylate; a 60 μM concentration of both resulted in half-maximal inhibition. No effect on enzyme activity was observed with either 1 mM oxaloacetate or 1 mM glyoxylate alone. Also, ATP, ADP, AMP, fructose-1,6-bisphosphate, glyceraldehyde-3-phosphate, 3-phosphoglycerate, phosphoenolpyruvate, pyruvate, or any other intermediate of the citric acid cycle at a concentration of 1 mM did not show an effect on ICD activity. Significant inhibition of the enzyme was observed at higher concentrations of oxaloacetate (K_i = 10 mM), 2-oxoglutarate (K_i = 12 mM), or citrate (K_i = 15 mM). The inhibition by these three metabolites, that by oxaloacetate plus glyoxylate, and also the K_m values correspond to data obtained with partially purified ICD from *C. glutamicum* subsp. *flavum* (39), suggesting that the ICDs of the two organisms are identical.

DISCUSSION

This paper describes the cloning and analysis of the *C. glutamicum* ICD gene and the biochemical characterization of the ICD enzyme. Our results show that the *C. glutamicum* ICD is a monomer which corresponds in its molecular weight to the known monomeric ICDs from *Vibrio* sp. strain ABE-1 (M_r = 80,500) (24, 25), *V. parahaemolyticus* (M_r = 80,000) (13), *R. vannielii* (M_r = 75,000) (32), and *A. vinelandii* (M_r = 80,000) (6). In its primary structure, the *C. glutamicum* ICD displays great similarity to the monomeric ICD-II from *Vibrio* sp. strain ABE-1 (25). Since the amino acid sequences of both the ICD-II from *Vibrio* sp. strain ABE-1 and the ICD from *C. glutamicum* do not show even short stretches similar to any known amino acid sequence of a dimeric ICD, it seems likely that two distinct ICDs, i.e., the monomeric type and the dimeric type, have evolved independently. With respect to the evolution of two distinct ICD enzymes, it is noteworthy that all organisms so far known to possess the monomeric type (i.e., *Vibrio* sp. strain ABE-1, *V. parahaemolyticus*, *R. vannielii*, and *A. vinelandii*) belong to the gram-negative purple bacteria. *C. glutamicum*, a gram-positive organism and thus phylogenetically far distant from the former group, is the first exception. Although data on ICD enzymes from gram-positive bacteria are scarce, the monomeric ICD is certainly not a general feature of gram-positive organisms, as it has been shown that the ICDs from an alkalophilic *Bacillus* sp. and from *Bacillus stearothermophilus* are dimeric (20, 49). However, it would be interesting to investigate whether other gram-positive bacteria also possess the monomeric type of ICD.

The monomeric ICD of *A. vinelandii* was previously inactivated by alkylation of a single methionine residue, and subsequently, an ICD-internal peptide containing the modified amino acid was isolated and sequenced (8). This peptide consists of the amino acid sequence G-K-S-A-L-R-K-F-I-A-A-Q-I-E-D-A-B-K-X-G-V-L-L-X-V-X-L-K-A-T-M-M, the last residue corresponding to the alkylated methionine (8). Comparison of this peptide sequence with that of the *C. glutamicum* ICD revealed a similar motif located between residues 226 and 257 in Fig. 4 and showing 46.4% identity. The last six amino acids are completely identical in the ICDs of *A. vinelandii*, *Vibrio* sp. strain ABE-1, and *C. glutamicum* (Fig. 4). These results sustain the high level of similarity between monomeric ICDs and support the idea that the alkylated methionine and/or the region close to it might be involved in the catalytic mechanism of the enzyme (8). Unfortunately, it is not possible to propose other important residues or regions by the alignment of the *Vibrio* sp. strain ABE-1 and the *C. glutamicum* ICDs, since both are very similar throughout the whole amino acid sequence (Fig. 4). In this context, it is worth mentioning that neither the *Vibrio* sp. strain ABE-1 nor the *C. glutamicum* ICD sequence contains the typical $NADP^+$-binding motif consisting of -**G**-X-**G**-X-X-**A**-X-X-X-**A**-X-X-X-X-X-X-**G**- which is found in several other $NADP^+$-dependent dehydrogenases (46), including the *C. glutamicum* GDH (4). With respect to substrate and coenzyme binding sites, it would certainly be of great interest to compare the three-dimensional structure of a monomeric ICD with that of a dimeric enzyme, e.g., that of *E. coli*, which has been studied in detail (22, 23, 51).

The specific activity of the *C. glutamicum* ICD was found to be independent of the growth substrate and the growth phase. In contrast, the ICD activity of several other organisms has been reported to vary when the cells are grown on different carbon sources. In enteric bacteria such as *E. coli*, *Klebsiella aerogenes*, *Salmonella typhimurium*, and *Serratia marcescens*, the specific ICD activity was found to be three- to fourfold lower during growth on acetate than during growth on glucose (2). Further studies with *E. coli* revealed that this change in specific activity is due to a reversible covalent modification of ICD by phosphorylation and dephosphorylation (14, 30, 38). In some other bacteria, e.g., *A. calcoaceticus* and *Vibrio* sp. strain ABE-1, it was found that the specific ICD activity increased when they were grown on acetate (24, 40), and in the case of the latter organism it was shown that this increase is due to transcriptional regulation of the gene for the dimeric ICD-I (25). Since the specific activity of ICD in *C. glutamicum* was the same under all conditions tested, we assume that in this organism the ICD gene is not under transcriptional or translational control and that the ICD enzyme is not regulated by a phosphorylation and dephosphorylation mechanism.

In *E. coli*, the phosphorylation system was found to keep the specific ICD activity at the WT level even when the *icd* gene from *E. coli* is overexpressed from plasmid and thus the level of ICD enzyme is raised more than 10-fold (31). The fact that *C. glutamicum* strains which harbor plasmids containing the homologous *icd* gene showed increased specific ICD activity and

thus no compensatory adaptation of the ICD activity sustains the above-mentioned assumption that this organism lacks the phosphorylation-dephosphorylation cycle of ICD. The absence of compensatory adaptation of the ICD activity may be the reason for the slower growth of the *icd*-overexpressing *C. glutamicum* strains. It can be speculated that in the recombinant strain, because of the 10-fold-higher ICD level, isocitrate is forced through the citric acid cycle, and thus insufficient isocitrate can enter the glyoxylate bypass. Since this bypass is essential for the biosynthesis of cellular substances during growth on acetate (29), a limitation of the carbon flux through the glyoxylate cycle would result in slower growth.

The *C. glutamicum* ICD was weakly inhibited by oxaloacetate, 2-oxoglutarate, and citrate. Although we do not know the exact intracellular concentrations of oxaloacetate, 2-oxoglutarate, and citrate in *C. glutamicum*, we regard the inhibition of ICD by these compounds in vivo as relatively unimportant because of the high K_i values. On the other hand, the ICD was severely inhibited in the presence of low concentrations of oxaloacetate and glyoxylate together. Concerted inhibition by these metabolites has been shown for most dimeric and monomeric $NADP^+$-dependent ICDs (5, 48). In the case of the *E. coli* ICD, the concentration of both oxaloacetate and glyoxylate needed for inhibition is relatively high (a 60 μM concentration of each would inhibit the enzyme by only 3% [37, 48]), and the binding of both to the enzyme was shown to be relatively slow (37). These results led to the conclusion that inhibition of the *E. coli* ICD by oxaloacetate and glyoxylate is physiologically not significant (37, 38). However, compared with the *E. coli* ICD, the *C. glutamicum* enzyme is much more sensitive to oxaloacetate and glyoxylate (a 60 μM concentration of each inhibited the enzyme by 50%), suggesting that the concerted inhibition by these metabolites in *C. glutamicum* might have physiological significance. Taking into account the facts that (i) the specific ICD activity in this organism is independent of the carbon source in the medium, (ii) during growth on acetate the specific activities of ICD (1 U/mg of protein) and isocitrate lyase (2.5 U/mg of protein [41]) are in the same order of magnitude, and (iii) the affinity of ICD for D$_S$-*threo*-isocitrate (K_m = 12 μM) is much higher than that of isocitrate lyase (K_m = 280 μM [41]), there should be an effective regulation of ICD activity to allow the operation of the glyoxylate cycle during growth on acetate. The concerted inhibition of ICD by oxaloacetate and glyoxylate would provide a possible mechanism for directing the carbon flux from the citric acid cycle to the glyoxylate cycle. In order to define the precise mechanism of the carbon flux control at the isocitrate branch point in *C. glutamicum*, further investigations are required.

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REFERENCES

1. **Apostolakos, D., P. A. Menter, B. J. Rampsch, H. C. Reeves, and E. A. Birge.** 1982. Genetic map position of the cistron coding for isocitrate dehydrogenase in *Escherichia coli* K-12. Curr. Microbiol. **7:**45–47.
2. **Bennett, P. M., and W. H. Holms.** 1975. Reversible inactivation of isocitrate dehydrogenase of *Escherichia coli* ML308 during growth on acetate. J. Gen. Microbiol. **87:**37–51.
3. **Birnboim, H. C.** 1983. A rapid alkaline extraction method for the isolation of plasmid DNA. Methods Enzymol. **100:**243–255.
4. **Börmann, E. R., B. J. Eikmanns, and H. Sahm.** 1992. Molecular analysis of the *Corynebacterium glutamicum gdh* gene encoding glutamate dehydrogenase. Mol. Microbiol. **6:**317–326.
5. **Chen, R. D., and P. Gadal.** 1990. Structure, functions, and regulation of NAD and NADP dependent isocitrate dehydrogenases in higher plants and in other organisms. Plant Physiol. Biochem. **28:**411–427.
6. **Chung, A. E., and J. S. Franzen.** 1969. Oxidized triphosphopyridine nucleotide specific isocitrate dehydrogenase from *Azotobacter vinelandii*: isolation and characterization. Biochemistry **8:**3175–3184.
7. **Cupp, J. R., and L. McAlister-Henn.** 1992. Cloning and characterization of the gene encoding the IDH1 subunit of NAD^+-dependent isocitrate dehydrogenase from *Saccharomyces cerevisiae*. J. Biol. Chem. **267:**16417–16423.
8. **Edwards, D. J., R. L. Henrikson, and A. E. Chung.** 1974. Triphosphopyridine nucleotide specific isocitrate dehydrogenase from *Azotobacter vinelandii*: alkylation of a specific methionine residue and amino acid sequence of the peptide containing this residue. Biochemistry **13:**677–683.
9. **Eikmanns, B. J.** 1992. Identification, sequence analysis, and expression of a *Corynebacterium glutamicum* gene cluster encoding the three glycolytic enzymes glyceraldehyde-3-phosphate dehydrogenase, 3-phosphoglycerate kinase, and triosephosphate isomerase. J. Bacteriol. **174:**6076–6086.
10. **Eikmanns, B. J., E. Kleinertz, W. Liebl, and H. Sahm.** 1991. A family of *Corynebacterium glutamicum*/*Escherichia coli* shuttle vectors for cloning, controlled gene expression and promoter probing. Gene **102:**93–98.
11. **Eikmanns, B. J., M. Metzger, D. Reinscheid, M. Kircher, and H. Sahm.** 1991. Amplification of three threonine biosynthesis genes in *Corynebacterium glutamicum* and its influence on carbon flux in different strains. Appl. Microbiol. Biotechnol. **34:**617–622.
12. **Eikmanns, B. J., N. Thum-Schmitz, L. Eggeling, K.-U. Lüdtke, and H. Sahm.** 1994. Nucleotide sequence, expression and transcriptional analysis of the *Corynebacterium glutamicum gltA* gene encoding citrate synthase. Microbiology **140:**1817–1828.
13. **Fukunaga, N., S. Imagawa, T. Sahara, A. Ishii, and M. Suzuki.** 1992. Purification and characterization of monomeric isocitrate dehydrogenase with $NADP^+$-specificity from *Vibrio parahaemolyticus*. J. Biochem. **112:**849–855.
14. **Garnak, M., and H. C. Reeves.** 1979. Phosphorylation of isocitrate dehydrogenase of *Escherichia coli*. Science **203:**1111–1112.
15. **Gornall, A. G., C. J. Bardawill, and M. M. David.** 1949. Determination of serum proteins by means of the Biuret reaction. J. Biol. Chem. **177:**751–766.
16. **Hanahan, D.** 1985. Techniques for transformation of E. coli, p. 109–135. *In* D. M. Glover (ed.), DNA cloning: a practical approach, vol. 1. IRL Press, Oxford.
17. **Haselbeck, R. J., R. F. Colman, and L. McAlister-Henn.** 1992. Isolation and sequence of a cDNA encoding porcine mitochondrial NADP-specific isocitrate dehydrogenase. Biochemistry **31:**6219–6223.
18. **Haselbeck, R. J., and L. McAlister-Henn.** 1991. Isolation, nucleotide sequence, and disruption of the *Saccharomyces cerevisiae* gene encoding mitochondral NADP(H)-specific isocitrate dehydrogenase. J. Biol. Chem. **266:**2339–2345.
19. **Hoischen, C., and R. Krämer.** 1989. Evidence of an efflux carrier system involved in the secretion of glutamate by *Corynebacterium glutamicum*. Arch. Microbiol. **151:**342–347.
20. **Howard, R. L., and R. R. Becker.** 1970. Isolation and some properties of the triphosphopyridine nucleotide isocitrate dehydrogenase from *Bacillus stearothermophilus*. J. Biol. Chem. **245:**3186–3194.
21. **Huh, T. L., J. H. Ryu, J. W. Huh, H. C. Sung, I. U. Oh, B. J. Song, and R. L. Veech.** 1993. Cloning of a cDNA encoding bovine mitochondrial $NADP^+$-specific isocitrate dehydrogenase and structural comparison with its isoenzymes from different species. Biochem. J. **292:**705–710.
22. **Hurley, J. H., A. M. Dean, D. E. Koshland, Jr., and R. M. Stroud.** 1991. Catalytic mechanism of $NADP^+$-dependent isocitrate dehydrogenase: implications from the structures of magnesium-isocitrate and $NADP^+$ complexes. Biochemistry **30:**8671–8678.
23. **Hurley, J. H., A. M. Dean, J. L. Sohl, D. E. Koshland, Jr., and R. M. Stroud.** 1990. Regulation of an enzyme by phosphorylation at the active site. Science **249:**1012–1016.
24. **Ishii, A., T. Ochiai, S. Imagawa, N. Fukunaga, S. Sasaki, O. Minowa, Y. Mizuno, and H. Shiokawa.** 1987. Isozymes of isocitrate dehydrogenase from an obligately psychrophilic bacterium, *Vibrio* sp. strain ABE-1: purification, and modulation of activities by growth conditions. J. Biochem. **102:**1489–1498.
25. **Ishii, A., M. Suzuki, T. Sahara, Y. Takada, S. Sasaki, and N. Fukunaga.** 1993. Genes encoding two isocitrate dehydrogenase isozymes of a psychrophilic bacterium, *Vibrio* sp. strain ABE-1. J. Bacteriol. **175:**6873–6890.
26. **Jin, S., and A. L. Sonenshein.** 1994. Identification of two distinct *Bacillus subtilis* citrate synthase genes. J. Bacteriol. **176:**4669–4679.
27. **Kinoshita, S.** 1985. Glutamic acid bacteria, p. 115–142. *In* A. L. Demain and N. A. Solomon (ed.), Biology of industrial microorganisms. Benjamin/Cummings Publishing Co., London.
28. **Kinoshita, S., and K. Tanaka.** 1972. Glutamic acid, p. 263–324. *In* K. Yamada (ed.), The microbial production of amino acids. John Wiley & Sons, Inc., New York.
29. **Kornberg, H. L.** 1966. Anaplerotic sequences and their role in metabolism. Essays Biochem. **2:**1–31.
30. **LaPorte, D. C.** 1993. The isocitrate dehydrogenase phosphorylation cycle: regulation and enzymology. J. Cell. Biochem. **51:**14–18.
31. **LaPorte, D. C., P. E. Thorsness, and D. E. Koshland, Jr.** 1985. Compensatory phosphorylation of isocitrate dehydrogenase: a mechanism for adapta-

tion to the intracellular environment. J. Biol. Chem. **260:**10563–10568.
32. **Leyland, M. L., and D. J. Kelly.** 1991. Purification and characterization of a monomeric isocitrate dehydrogenase with dual coenzyme specificity from the photosynthetic bacterium *Rhodomicrobium vannielii*. Eur. J. Biochem. **202:** 85–93.
33. **Liebl, W., A. Bayerl, B. Schein, U. Stillner, and K. H. Schleifer.** 1989. High efficiency electroporation of intact Corynebacterium glutamicum cells. FEMS Microbiol. Lett. **65:**299–304.
34. **Miyazaki, K., H. Eguchi, A. Yamagishi, T. Wakagi, and T. Oshima.** 1992. Molecular cloning of the isocitrate dehydrogenase gene of an extreme thermophile, *Thermus thermophilus* HB8. Appl. Environ. Microbiol. **58:**93–98.
35. **Muro-Pastor, M. I., and F. J. Florencio.** 1994. $NADP^+$-isocitrate dehydrogenase from the cyanobacterium *Anabaena* sp. strain PCC7120: purification and characterization of the enzyme and cloning, sequencing, and disruption of the *icd* gene. J. Bacteriol. **176:**2718–2726.
36. **Nachlas, M. M., M. B. Davidson, J. D. Goldberg, and A. M. Seligman.** 1963. Colorimetric method for the measurement of isocitric dehydrogenase activity. J. Lab. Clin. Med. **62:**148–158.
37. **Nimmo, H. G.** 1986. Kinetic mechanism of *Escherichia coli* isocitrate dehydrogenase and its inhibition by glyoxylate and oxaloacetate. Biochem. J. **234:**317–323.
38. **Nimmo, H. G.** 1987. The tricarboxylic acid cycle and anaplerotic reactions, p. 156–169. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
39. **Ozaki, H., and I. Shiio.** 1968. Regulation of the TCA and glyoxylate cycles in *Brevibacterium flavum*. J. Biochem. **64:**355–363.
40. **Reeves, H. C., S. O'Neil, and P. D. J. Weitzmann.** 1986. Changes in NADP-isocitrate dehydrogenase isoenzyme levels in *Acinetobacter calcoaceticus* in response to acetate. FEMS Microbiol. Lett. **35:**229–232.
41. **Reinscheid, D. J., B. J. Eikmanns, and H. Sahm.** 1994. Characterization of the isocitrate lyase gene from *Corynebacterium glutamicum* and biochemical analysis of the enzyme. J. Bacteriol. **176:**3474–3483.
42. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
43. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
44. **Schäfer, A., J. Kalinowski, R. Simon, A. H. Seep-Feldhaus, and A. Pühler.** 1990. High-frequency conjugal plasmid transfer from gram-negative *Escherichia coli* to various gram-positive coryneform bacteria. J. Bacteriol. **172:** 1663–1666.
45. **Schwarzer, A., and A. Pühler.** 1991. Manipulation of *Corynebacterium glutamicum* by gene disruption and replacement. Bio/Technology **9:**84–87.
46. **Scrutton, N. S., A. Berry, and R. N. Perham.** 1990. Redesign of the coenzyme specificity of a dehydrogenase by protein engineering. Nature (London) **343:**38–43.
47. **Self, C. H., and P. D. J. Weitzman.** 1972. The isocitrate dehydrogenases of *Acinetobacter lwoffi*. Biochem. J. **130:**211–219.
48. **Shiio, I., and H. Ozaki.** 1968. Concerted inhibition of isocitrate dehydrogenase by glyoxylate plus oxaloacetate. J. Biochem. **64:**45–53.
49. **Shikata, S., K. Ozaki, S. Kawai, S. Ito, and K. Okamato.** 1988. Purification and characterization of $NADP^+$-linked isocitrate dehydrogenase from an alkalophilic *Bacillus*. Biochim. Biophys. Acta **952:**282–289.
50. **Simon, R., U. Priefer, and A. Pühler.** 1983. A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in gram negative bacteria. Bio/Technology **1:**784–791.
51. **Stoddard, B. L., and D. E. Koshland, Jr.** 1993. Structure of isocitrate dehydrogenase with α-ketoglutarate at 2.7-Å resolution: conformational changes induced by decarboxylation of isocitrate. Biochemistry **32:**9317–9322.
52. **Thorsness, P. E., and D. E. Koshland, Jr.** 1987. Inactivation of isocitrate dehydrogenase by phosphorylation is mediated by the negative charge of the phosphate. J. Biol. Chem. **262:**10422–10425.
53. **Tinoco, I., P. N. Borer, B. Dengler, M. D. Levine, O. C. Uhlenbeck, D. M. Crothers, and J. Gralla.** 1973. Improved estimation of secondary structure in ribonucleic acid. Nature (London) New Biol. **246:**40–41.
54. **Udvardi, M. K., T. R. McDermott, and M. L. Kahn.** 1993. Isolation and characterization of a cDNA encoding $NADP^+$-specific isocitrate dehydrogenase from soybean (*Glycine max*). Plant Mol. Biol. **21:**739–752.
55. **Vanni, P., E. Giachetti, G. Pinzauti, and B. A. McFadden.** 1990. Comparative structure, function and regulation of isocitrate lyase, an important assimilatory enzyme. Comp. Biochem. Physiol. **95B:**431–458.
56. **Vieira, J., and J. Messing.** 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene **19:**259–268.
57. **Walsh, K., and D. E. Koshland, Jr.** 1985. Branch point control by the phosphorylation state of isocitrate dehydrogenase. J. Biol. Chem. **260:**8430–8437.