Duck liver ‘malic’ enzyme

Expression in *Escherichia coli* and characterization of the wild-type enzyme and site-directed mutants

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A cDNA for duck liver ‘malic’ enzyme (EC 1.1.1.40) was subcloned into pUC-8, and the active enzyme was expressed in *Escherichia coli* TG-2 cells as a fusion protein including a 15-residue *N*-terminal leader from β -galactosidase coded by the *lacZ'* gene. C99S and R70Q mutants of the enzyme were generated by the M13 mismatch technique. The recombinant enzymes were purified to near homogeneity by a simple two-step procedure and characterized relative to the enzyme isolated from duck liver. The natural duck enzyme has a subunit molecular mass of approx. 65 kDa, and the following kinetic parameters for oxidative decarboxylation of L-malate at pH 7.0: K_m NADP⁺ (4.6 μ M); K_m L-malate (73 μ M); k_{cat} (160 s⁻¹); K_a (2.4 μ M) and K_a' (270 μ M), dissociation constants of Mn²⁺ at ‘tight’ (activating) and ‘weak’ metal sites; and substrate inhibition (51 % of k_{cat} at 8 mM-L-malate). Properties of the *E. coli*-derived recombinant wild-type enzyme are indistinguishable from those of the natural duck enzyme. Kinetic parameters of the R70Q mutant are relatively unaltered, indicating that Arg-70 is not required for the reaction. The C99S mutant has unchanged K_m for NADP⁺ and parameters for the ‘weak’ sites (i.e. inhibition by L-malate, K_a'); however, k_{cat} decreased 3-fold and K_m for L-malate and K_a each increased 4-fold, resulting in a catalytic efficiency [$k_{cat}/(K_m \text{ NADP}^+ \times K_m \text{ L-malate} \times K_a)$] equal to 3.7% of the natural duck enzyme. These results suggest that the positioning of Cys-99 in the sequence is important for proper binding of L-malate and bivalent metal ions.

INTRODUCTION

Hepatic ‘malic’ enzyme is a tetrameric molecule with identical subunits (Nevaldine *et al.*, 1974). It catalyses oxidative decarboxylation of L-malate to CO₂ and pyruvate and, in addition, the following bivalent-metal-ion-dependent NADP⁺(H)-linked component reactions: decarboxylation of oxalacetate; exchange of protons between pyruvate and medium water; and, at much lower rates, reduction of a broad spectrum of α -oxo mono- and di-carboxylic acids to the corresponding α -hydroxy acids (Hsu, 1982; Bratcher & Hsu, 1982).

Chemical modification of the pigeon liver enzyme with group-specific reagents suggests the presence of an arginine(s) at the substrate site positioned for ion pairing with C-1 of L-malate (Vernon & Hsu, 1983). A histidine residue(s) is located at the nucleotide site (Chang & Hsu, 1977a). Its involvement in NADPH binding is implicated by direct titration experiments, which also suggest the participation of this residue as a proton sink in the catalytic reaction (Pry & Hsu, 1980). A cysteine residue is positioned near the malate site located at the interface between neighbouring subunits (Hsu, 1982; Satterlee & Hsu, 1991). Alkylation of this residue by the affinity label bromopyruvate causes loss of oxidative decarboxylase activity with half-of-the-sites reactivity; the reductase activity on pyruvate, however, is enhanced 1.5-fold (Chang & Hsu, 1977b; Pry & Hsu, 1978).

Binding of NADPH is unaffected, whereas binding of L-malate is abolished (Satterlee & Hsu, 1991).

Half-of-the-sites behaviour was also suggested by results of kinetic and equilibrium substrate-binding experiments (Pry & Hsu, 1980; Hsu & Pry, 1980). In the oxidative decarboxylase reaction, L-malate exhibits Michaelis–Menten kinetics at low concentrations corresponding to binding at two high-affinity (‘tight’) sites. At higher concentrations, the substrate binds weakly to two remaining sites on the enzyme tetramer and inhibits the reaction; this inhibition is relieved by high concentrations of Mn²⁺ occupying both ‘tight’ and low-affinity (‘weak’) metal sites.

In the present paper, we report the nucleotide sequence of a cDNA for duck liver ‘malic’ enzyme and its deduced amino acid sequence. A simple two-step procedure was devised for the purification of the recombinant wild-type enzyme and two site-directed mutants (C99S and R70Q) from overproducing strains of *Escherichia coli*. The steady-state kinetic properties of the natural duck liver enzyme and recombinant ‘malic’ enzymes were determined and compared.

MATERIALS AND METHODS

Materials

Biochemical reagents of the highest grade available were

Abbreviation used: IPTG, isopropyl β -D-thiogalactopyranoside.

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The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases.

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obtained from commercial sources. Restriction endonucleases, T_4 DNA ligase, polynucleotide kinase and DNA polymerase I (Klenow fragment) were purchased from Boehringer Corp., and used according to the manufacturer's instructions. [γ - 32 P]ATP was from Amersham International. Distilled water, further purified through a deionizer system, was used throughout. 'Malic' enzyme was purified from duck liver by the method of Satterlee & Hsu (1991).

Methods

Oxidative decarboxylase and pyruvate reductase activities were assayed at pH 7.4 according to published procedures (Hsu & Lardy, 1967; Tang & Hsu, 1974). A unit of activity is defined as the conversion of 1 μ mol of NADP⁺(H)/min at 30 °C. Kinetic parameters were determined in assays performed essentially as described by Hsu & Pry (1980) at variable L-malate, MnCl₂ or NADP⁺ concentrations and pH 7.0, in a volume of 1 ml. A subunit molecular mass for the 'malic' enzyme from pigeon liver (65 kDa; Nevaldine *et al.*, 1974) was used for the duck enzyme in all calculations. This value was confirmed by comparing the mobility of the duck enzyme with the 67 kDa BSA on SDS/PAGE. Protein concentration was determined by the dye-binding method (Bradford, 1976). DNA manipulations were carried out essentially according to Sambrook *et al.* (1989). Amino acid sequence analysis was performed in an Applied Biosystems gas phase sequencer model 470A (Hunkapiller *et al.*, 1986).

Subcloning of 'malic' enzyme cDNA into pUC-8

pDME-1 (Fig. 1a) contains the cDNA for duck liver 'malic' enzyme cloned into the *Pst*I site of pBR322 (Glynias *et al.*, 1984). It was digested with *Pst*I and the products were separated by agarose-gel electrophoresis. The 1.9 kb insert containing 'malic' enzyme cDNA was excised, extracted by solvent partitioning, and ligated with similarly digested gel-purified pUC-8 DNA. Competent *E. coli* TG-2 cells were transformed with the ligation product (pHF-1, Fig. 1b) by electroporation, and plated on 2 $\times$ YT agar plates with 5-bromo-4-chloro-3-indolyl- β -D-galacto-

side (0.1 mM), isopropyl β -D-thiogalactopyranoside (IPTG; 0.15 mM) and ampicillin (100 μ g/ml). Individual β -galactosidase-negative colonies containing the insert were grown overnight in 2 $\times$ YT (100 μ g of ampicillin/ml) liquid cultures to obtain expression of the enzyme.

Nucleotide sequence of duck liver 'malic' enzyme cDNA

Initial DNA sequence analysis was carried out by the chain-termination method (Sanger *et al.*, 1977) using M13 subclones of pDME-1 single-stranded DNA and the universal primer. This analysis indicated that the entire protein-coding region was contained within the *Pst*I restriction fragment of pDME-1. To confirm the initial result and to complete the sequence from both strands, the nucleotide sequence of the insert DNA of pHF-1 was determined by using double-stranded DNA and a DuPont Genesis 2000 automated sequencer. A set of ten oligonucleotide primers was made on a DuPont CODER 300 DNA synthesizer by using the initial sequence data. For the forward strand, the primers were: reverse universal; 5'-TCGAAATGAAAAGCT-CTTCTAT-3'; 5'-TCGGCTGCTATGGCATGGGCA-3'; 5'-TTGCTAATGCCAATGCATTCCG-3'; 5'-AATATGGATGGTTGACTCAAAG-3'; 5'-AGCAATCCTACCAGCAAA-GC-3'; and 5'-GTCGCCTGTACCCTCCTTTAGT-3'. Those used to sequence the reverse strand were: universal; 5'-TGT-AGGCTTCCTCTGCGATC-3'; 5'-TGCTTTGCTGGTAGG-ATTGC-3'; 5'-CCATAGCCATGACAATGAGG-3'; 5'-GAT-TCTCTTGTGTCTCAGCC-3'; and 5'-GACCGAACGGTGC-TTATAC-3'. The sequencing strategies are shown in Fig. 2.

Oligonucleotide-directed mutagenesis in M13mp19

'Malic' enzyme mutants were generated by the oligonucleotide-directed mismatch procedure in M13 (Winter *et al.*, 1982) with the appropriate mutagenic oligonucleotides as primers for extensions *in vitro*. Recombinant M13 phage DNA was obtained by subcloning the 'malic' enzyme cDNA into the polylinker of M13mp19 between *Bam*HI and *Hind*III sites. After electroporation, the transformants were used to prepare single-stranded DNA. The single-stranded DNA was annealed to

Fig. 1. Plasmid constructs (a) pDME-1 and (b) pHF-1

The cross-hatched area in pDME-1 is the *Pst*I fragment containing 'malic' enzyme cDNA insert. In pHF-1, the *Pst*I fragment (DME) and ampicillin-resistance gene (AMP) are both cross-hatched.

Fig. 2. Sequencing strategy for the 'malic' enzyme cDNA

Restriction fragments of the insert DNA of pDME-1 were subcloned into the multiple cloning sites of M13mp10, 11, 18 or 19, and nucleotide sequences of the resulting single-stranded DNA determined by the chain-termination method (Sanger *et al.*, 1977) using the universal primer (a). The *Pst*I fragment of pHF-1 was sequenced on both strands using double-stranded DNA and the primers described in the Materials and methods section (b). The arrows indicate the directions and extent of sequencing of each restriction fragment or from each primer used.

mutant primers phosphorylated at their 5' ends, extended by the Klenow fragment of DNA polymerase I, and ligated by *T*₄ DNA ligase. The resultant product was used to transform competent *E. coli* BMH 71-18 *mutL* cells by the CaCl_2 method. Colonies grown from overnight cultures were then subjected to screening with γ -³²P-labelled primer (Carter *et al.*, 1985). Phage DNA (RF form) was isolated from positive colonies and the mutant 'malic' enzyme cDNAs were then subcloned into pUC-8.

The following mutants (underlined) were isolated: C99S (primer sequence 5'-GACTGGCTTCACAGCAATAT-3') and R70Q (primer sequence 5'-TTCAGGATCAGAAATGAAAAG-3'). The active-site cysteine was identified as residue 99 (Satterlee & Hsu, 1991) by labelling the enzyme with 3-bromo-[1-¹⁴C]pyruvate, and aligning the sequence of the chromatographically purified radioactive tryptic peptide with that of the natural duck enzyme. [7-¹⁴C]Phenylglyoxal was used to label the

arginine at the malate site (Vernon & Hsu, 1983). However, a proteinase digest of the labelled enzyme yielded multiple radio-active peptides (Y. S. Wang & R. Y. Hsu, unpublished work). The major peptide contained Arg-70 and was tested in this study.

Purification of *E. coli*-derived 'malic' enzymes

Recombinant 'malic' enzymes were purified from 2- to 4-litre TG-2 cultures grown in 0.5 mM-IPTG. The cells were pelleted, resuspended in 0.05 vol. of 0.1 M-triethanolamine, pH 7.4, and sonicated at 0 °C by four 20 s bursts from the microprobe of a w-375 sonicator (Heat System-Ultrasonics Inc., Melville, NY, U.S.A.) at an output setting of 2. After centrifugation, the extract (F-1) was made 0.1 M in magnesium acetate, acidified to pH 5.5 with cold 1 M-acetic acid, and heated for 3 min in a 58 °C bath. Denatured proteins were removed by centrifugation (10000 *g* for 10 min). The supernatant fraction (F-2) was dialysed against 20 mM-Tris/HCl buffer, pH 8.0, overnight, and chromatographed on a 150 ml Pharmacia Q-Sepharose column pre-equilibrated with the same buffer. After washing, 'malic' enzyme was eluted with the same buffer containing 20 mM-magnesium acetate. Enzymically active fractions were combined (F-3), concentrated by precipitation with 70 % -satd. $(\text{NH}_4)_2\text{SO}_4$, and stored at -70 °C in a minimum volume (2-3 ml) of dialysis buffer containing 10 % (v/v) glycerol and 2 mM-dithiothreitol.

RESULTS

Expression of the duck liver 'malic' enzyme in *E. coli*

The 'malic' enzyme cDNA was transferred from the pBR322-derived pDME-1 to pUC-8 for expression of the wild-type enzyme. pUC-8 was chosen as the expression vector because it has a high copy number, and because the *Pst*I site in its multiple cloning region aligned the 'malic' enzyme insert in the reading frame with the start site for β -galactosidase. Constructs with the insert in coding (pHF-1; Fig. 1b) and non-coding (pHF-2; not shown) orientations, yielding *Eco*RI restriction fragments of 0.7 + 3.8 kb and 1.2 + 3.3 kb respectively were obtained. In parallel experiments, protein patterns of overnight growths of *E. coli* TG-2 cells transformed with these plasmids were examined by SDS/PAGE. As shown in Fig. 3, TG-2 (pHF-1) cells expressed the 65 kDa 'malic' enzyme subunit in significantly higher amounts than that seen in control TG-2 (pUC-8) cells, or TG-2 (pHF-2) cells with the reverse orientation (not shown). Furthermore, expression of the enzyme in the former was enhanced by inducing the *lacZ'* gene with 0.5-1.0 mM-IPTG.

A Cys-99 mutant with conservative replacement (C99S) and an Arg-70 mutant with neutral replacement (R70Q) were prepared, and confirmed by nucleotide sequence analysis across the mutation sites using appropriate primers. SDS/PAGE analysis of total cellular proteins showed that for each mutant, the 65 kDa 'malic' enzyme subunit band was over-expressed.

Oxidative decarboxylase activity was determined on supernatant fractions (F-1) of sonicated cells from overnight cultures as described in the Materials and methods section. TG-2 (pHF-1) cells yielded 438 units of activity/l of culture, whereas control TG-2 (pUC-8) cells had negligible (< 10 units/l of culture) activity. The former value was increased to 576 units/l by 0.2 mM-IPTG, and 656-725 units/l by 0.5 mM-IPTG, or 1 mM-IPTG added 40 min before the cells were harvested. In comparison, cells harbouring the R70Q and C99S mutants yielded lower values of 604 and 380 units/l of culture respectively in the presence of 0.5 mM-IPTG. Purified 'malic' enzyme from duck liver has a specific activity of 30-40 units/mg of protein. On the basis of this value, TG-2 (pHF-1) cultures are calculated to yield 18-24 mg of recombinant wild-type enzyme/l.

The nucleotide sequence of 'malic' enzyme cDNA and the

Fig. 3. SDS/PAGE (Phastgel) of total cellular proteins

E. coli TG-2 cells were obtained from overnight cultures grown in $2 \times$ YT medium (100 μ g of ampicillin/ml) and IPTG as indicated. The cells were pelleted, and heated to 100 °C for 10 min in 2.5% SDS before electrophoresis in 12.5% acrylamide gel. Lane 1, BSA (1 mg/ml, 67 kDa); lanes 2, 3, 4, TG-2 (pUC-8) cells grown in the presence of 0, 0.5 μ M- and 1.0 mM-IPTG respectively; lanes 5, 6, 7, TG-2 (pHF-1) cells grown in the presence of 0, 0.5 mM- and 1.0 mM-IPTG respectively; lane 8, duck liver 'malic' enzyme (1 mg/ml).

deduced amino acid sequence of the protein are shown in Fig. 4. *N*-Terminal sequence analyses revealed that the *N*-terminus of the natural duck liver enzyme is blocked; the recombinant wild-type enzyme has a 15-residue leader sequence encoded by the *lacZ'* gene preceding the deduced *N*-terminal methionine. The predicted subunit molecular masses of the natural and fusion enzymes are 61.9 and 63.5 kDa respectively.

Purification and characterization of *E. coli*-derived 'malic' enzymes

The relatively high level of expression of 'malic' enzyme in *E. coli* transformed with the wild-type cDNA permitted the rapid isolation of this protein by a simple two-step procedure using heat denaturation and anion-exchange chromatography on a Pharmacia Q-Sepharose column. This procedure was also used to obtain the mutant enzymes. Specific activities of 30–45, 28 and 6.3–8.1 units/mg of protein were obtained for purified (F-3) recombinant wild-type enzyme and R70Q and C99S mutants, representing 100, 93 and 21–27% respectively of that of the enzyme purified from duck liver (Table 1). These enzyme preparations yielded near-homogeneous proteins of molecular mass approx. 65 kDa (Fig. 5). On the basis of a purification factor of 11–17-fold, the recombinant enzymes represent 6–9% of protein in the *E. coli* extract.

Purified enzymes were assayed for their abilities to reduce pyruvate to L-lactate. Similar values of 0.27, 0.20, 0.19 and 0.21 unit/mg of protein were obtained for the natural duck, recombinant wild-type, R70Q and C99S enzymes, corresponding to 0.9, 0.6, 0.8 and 3.3% of their oxidative decarboxylase activities respectively. Except for C99S, these values are comparable with

the value of 0.8% for the pigeon liver enzyme (Tang & Hsu, 1973). The *E. coli*-derived enzymes were inhibited 80–100% by 10 μ l of rabbit antiserum to the pigeon enzyme added to the 1 ml assay mixture, indicating their avian origin. The natural duck liver, recombinant wild-type and R70Q enzymes were inactivated 91–100% by incubation with 130 μ M-5,5'-dithiobis-(2-nitrobenzoic acid) for 10 min at room temperature and pH 7.4. The C99S mutant lacked the susceptible cysteine, and retained 94% activity under the same conditions. The R70Q mutant, however, was inactivated by 10 mM-phenylglyoxal in 100 mM-NaHCO₃ buffer, pH 7.5, and room temperature, with a pseudo-first-order rate constant (0.11 min⁻¹) comparable with that found for the recombinant wild-type enzyme (0.10 min⁻¹) and natural duck enzyme (0.12 min⁻¹; Vernon & Hsu, 1983).

Thermostability was examined by inactivating the enzyme (0.77 μ M) at 52 °C in 0.1 M-triethanolamine buffer, pH 7.8. First-order rate constants obtained from plots of the natural logarithms of residual oxidative decarboxylase activity versus time for the natural duck, recombinant wild-type, R70Q and C99S enzymes were 0.01, 0.016, 0.01 and 0.016 min⁻¹ respectively.

Steady-state parameters of oxidative decarboxylase activity of the natural duck enzyme were determined at pH 7.0. The reciprocal plot for varying NADP⁺ at 100 μ M-MnCl₂ and 3 mM-L-malate (Fig. 6a) was linear and yielded a *K_m* of 4.6 μ M for the nucleotide. L-Malate and Mn²⁺ each gave biphasic plots, and required the use of broad concentration ranges (7 μ M–8 mM for L-malate and 5 μ M–10 mM for MnCl₂) for proper definition. The plot in which L-malate was varied (Fig. 6b) at 100 μ M-MnCl₂ and saturating (100 μ M) NADP⁺ was linear from 7–60 μ M, but curved upwards with higher concentrations (> 360 μ M) because of

Fig. 4. Nucleotide and amino acid sequences

Arg-70, -107, -119, the tryptic peptide containing Cys-99 (Satterlee & Hsu, 1991), and the CTGCAG *Pst*I site are underlined. The sequence for the 15-residue leader is enclosed in brackets.

		[ACC ATG ATT ACG AAT TCC CGG GGA TCC GTC GAC <u>CTG CAG</u> CYC ACC]																				
		[Thr Met Ile Thr Asn Ser Arg Gly Ser Val Asp Leu Gln Leu Thr]																				
1	ATG AAG AGG GGC TAC GAG GTG CTC CGC GAC CCT CAC CTC AAC AAG GGA ATG GCA TTT ACC	60																				
1	Met Lys Arg Gly Tyr Glu Val Leu Arg Asp Pro His Leu Asn Lys Gly Met Ala Phe Thr	20																				
61	TTG GAG GAG AGG CAA CAA CTG AAT ATT CAT GGA CTA TTG CCA CCT TGC TTT CTC GGT CAA	120																				
21	Leu Glu Glu Arg Gln Gln Leu Asn Ile His Gly Leu Leu Pro Pro Cys Phe Leu Gly Gln	40																				
121	GAT GTC CAG GTT TTC AGT ATT CTT AAG AAT TTT GAA AGA CTG ACA TCC GAC CTA GAC AGA	180																				
41	Asp Val Gln Val Phe Ser Ile Leu Lys Asn Phe Glu Arg Leu Thr Ser Asp Leu Asp Arg	60																				
181	TAC ATT CTA CTA ATG AGT CTT CAG GAT CGA AAT GAA AAG CTC TTC TAT AAA GTG CTG ACT	240																				
61	Tyr Ile Leu Leu Met Ser Leu Gln Asp Arg Asn Glu Lys Leu Phe Tyr Lys Val Leu Thr	80																				
241	TCT GAC ATA GAA AGG TTC ATG CCT ATT GTT TAT ACT CCC ACT GTG GGA CTG GCT TGC CAG	300																				
81	Ser Asp Ile Glu Arg <u>Phe Met Pro Ile Val Tyr Thr Pro Thr Val Gly Leu Ala Cys Gln</u>	100																				
301	CAA TAT GGT TTA GCA TTT CGG AGG CCA AGA GGA CTT TTT ATT ACT ATC CAT GAC CGG GGA	360																				
101	<u>Gln Tyr Gly Leu Ala Phe Arg</u> Arg Pro Arg Gly Leu Phe Ile Thr Ile His Asp <u>Arg</u> Gly	120																				
361	CAC ATC GCT ACA ATG CTC AAG TCC TGG CCA GAA AGT GTC ATC AAG GCT ATT GTG GTG ACA	420																				
121	His Ile Ala Thr Met Leu Lys Ser Trp Pro Glu Ser Val Ile Lys Ala Ile Val Val Thr	140																				
421	GAT GGA GAA CGT ATT CTT GGT CTT GGA GAC CTC GGC TGC TAT GGC ATG GGC ATC CCG GTT	480																				
141	Asp Gly Glu Arg Ile Leu <u>Gly Leu Gly Asp Leu Gly</u> Cys Tyr Gly Met Gly Ile Pro Val	160																				
481	GGT AAA CTG GCA CTT TAT ACC GCG TGT GGA GGA GTG AAA CCT CAT GAG TGC CTA CCA GTG	540																				
161	Gly Lys Leu Ala Leu Tyr Thr Ala Cys Gly Gly Val Lys Pro His Glu Cys Leu Pro Val	180																				
541	ATG TTG GAT GTG GGA ACA GAT AAT GAG GCA CTG CTG AAA GAT CCT TTG TAT ATT GGG CTG	600																				
181	Met Leu Asp Val Gly Thr Asp Asn Glu Ala Leu Leu Lys Asp Pro Leu Tyr Ile Gly Leu	200																				
601	AGA CAC AAG AGA ATC AGA GGT CAA GCC TAT GAT GAT CTC CTT GAT GAA TTC ATG GAA GCT	660																				
201	Arg His Lys Arg Ile Arg Gly Gln Ala Tyr Asp Asp Leu Leu Asp Glu Phe Met Glu Ala	220																				
661	GTG ACT TCA AGG TAT GGC ATG AAC TGC CTC ATT CAA TTT GAG GAT TTT GCT AAT GCC AAT	720																				
221	Val Thr Ser Arg Tyr Gly Met Asn Cys Leu Ile Gln Phe Glu Asp Phe Ala Asn Ala Asn	240																				
721	GCA TTC CGC CTC CTG CAT AAG TAC CGT AAC AAG TAT TGC ACT TTC AAT GAT GAC ATT CAA	780																				
241	Ala Phe Arg Leu Leu His Lys Tyr Arg Asn Lys Tyr Cys Thr Phe Asn Asp Asp Ile Gln	260																				
781	GGA ACT GCA TCT GTG GCT GTT GCA GGT CTT CTT GCT GCT CTG CGT ATC ACT AAG AAC AGG	840																				
261	Gly Thr Ala Ser Val Ala Val Ala Gly Leu Leu Ala Ala Leu Arg Ile Thr Lys Asn Arg	280																				
841	CTA TCA GAT CAC ACA GTG CTG TTC CAG GGG GCT GGA GAA GCT GCG CTG GGG ATA GCA AAC	900																				
281	Leu Ser Asp His Thr Val Leu Phe Gln Gly Ala Gly Glu Ala Ala Leu Gly Ile Ala Asn	300																				
901	CTC ATT GTC ATG GCT ATG GAA AAA GAA GGA GTA TCT AAA GAG GCA GCT GTC AAA AGA ATA	960																				
301	Leu Ile Val Met Ala Met Glu Lys Glu Gly Val Ser Lys Glu Ala Ala Val Lys Arg Ile	320																				
961	TGG ATG GTT GAC TCA AAG GGC TTG ATA GTG AAG GGG CGT GCC TCA TTG ACA GCT GAG AAA	1020																				
321	Trp Met Val Asp Ser Lys Gly Leu Ile Val Lys Gly Arg Ala Ser Leu Thr Ala Glu Lys	340																				
1021	ACG CGA TTT GCA CAT GAA CAT GCT GAA ATG AAA AAT CTA GAA GAT ATT GTT AAA GAT ATA	1080																				
341	Thr Arg Phe Ala His Glu His Ala Glu Met Lys Asn Leu Glu Asp Ile Val Lys Asp Ile	360																				
1081	AAA CCA TCT GTG TTA ATA GGA GTT GCT GCA ATT GGT GGT GCT TTT ACT AAA GAG ATT CTT	1140																				
361	Lys Pro Ser Val Leu Ile Gly Val Ala Ala Ile Gly Gly Ala Phe Thr Lys Glu Ile Leu	380																				
1141	CAG GAT ATG GCT GCT TTC AAC AAA CGT CCT ATT ATT TTT GCT CTC AGC AAT CCT ACC AGC	1200																				
381	Gln Asp Met Ala Ala Phe Asn Lys Arg Pro Ile Ile Phe Ala Leu Ser Asn Pro Thr Ser	400																				
1201	AAA GCA GAG TGC ACT GCT GAG CAG TGC TAC AAA TAC ACA GAG GGA CGT GGT ATT TTT GCC	1260																				
401	Lys Ala Glu Cys Thr Ala Glu Gln Cys Tyr Lys Tyr Thr Glu Gly Arg Gly Ile Phe Ala	420																				
1261	AGT GGA AGT CCT TTT GAT CCT GTC ACT CTT CCA AAC GGA AAA ACT CTG TAT CCT GGA CAG	1320																				
421	Ser Gly Ser Pro Phe Asp Pro Val Thr Leu Pro Asn Gly Lys Thr Leu Tyr Pro Gly Gln	440																				
1321	GGT AAC AAT TCC TAT GTG TTC CCA GGA GTT GCC CTT GGT GTT ATT GCA TGC GGT CTG AAA	1380																				
441	Gly Asn Asn Ser Tyr Val Phe Pro Gly Val Ala Leu Gly Val Ile Ala Cys Gly Leu Lys	460																				
1381	CAT ATC GGC GAG GAT GTT TTC CTC ACA ACT GCT GAG GTA ATA GCT GAG CAA GTT TCA GAA	1440																				
461	His Ile Gly Glu Asp Val Phe Leu Thr Thr Ala Glu Val Ile Ala Glu Gln Val Ser Glu	480																				
1441	GAG AAT CTA CAG GAA GGT CGC CTG TAC CCT CCT TTA GTC ACA ATT CAG CAC GTG TCA CTG	1500																				
481	Glu Asn Leu Gln Glu Gly Arg Leu Tyr Pro Pro Leu Val Thr Ile Gln His Val Ser Leu	500																				
1501	AAA ATC GCT GTG AGG ATC GCA GAG GAA GCC TAC AGG AAT AAC ACT GCC TCC ACC TAT CCT	1560																				
501	Lys Ile Ala Val Arg Ile Ala Glu Glu Ala Tyr Arg Asn Asn Thr Ala Ser Thr Tyr Pro	520																				
1561	CAG CCC AAG GAC CTA GAA GCC TTC ATC CAG TCT CAG ATT TAC AGC ACT GAT TAT AAC AGC	1620																				
521	Gln Pro Lys Asp Leu Glu Ala Phe Ile Gln Ser Gln Ile Tyr Ser Thr Asp Tyr Asn Ser	540																				
1621	TTC GTG GCT GAC TCA TAT ACC TGG CCT GAA GAA GCG ATG AAA GTG AAA CTG TAA	1674																				
541	Phe Val Ala Asp Ser Tyr Thr Trp Pro Glu Glu Ala Met Lys Val Lys Leu ***	557																				

substrate inhibition. This inhibition was relieved by MnCl_2 at concentrations greater than $150 \mu\text{M}$, as indicated by the concave-downward plot for varying metal ion concentration at saturating, but partially inhibitory, (3 mM) L-malate (Fig. 6c). k_{cat} , K_m of NADP^+ and parameters associated with binding at 'tight' (K_m of L-malate and K_a) and 'weak' (inhibition by 8 mM-L-malate and K_a') sites are shown in Table 2. As expected, these values are similar to those reported for the pigeon liver enzyme (Hsu & Pry, 1980).

Linear NADP^+ and biphasic L-malate and MnCl_2 plots were also obtained for the *E. coli*-derived enzymes (not shown). As shown in Table 2, kinetic parameters of the recombinant wild-type enzyme were indistinguishable from those of the natural duck enzyme despite the presence of N-terminal leader. Parameters of the R70Q mutant were similar, except for a

slightly lower k_{cat} and higher K_m for L-malate and higher K_a . For the C99S mutant, binding of Mn^{2+} and L-malate at 'weak' sites (i.e. inhibition by L-malate, K_a') was essentially unchanged. K_m for NADP^+ was unchanged, whereas k_{cat} decreased 3-fold, and K_m for L-malate and K_a each increased 4-fold. Catalytic efficiencies of the R70Q and C99S mutants as defined by the ratio of $k_{\text{cat}}/(K_m \text{ NADP}^+ \times K_m \text{ L-malate} \times K_a)$ were respectively 30 and 3.7% of that for the natural enzyme.

DISCUSSION

The amino acid sequences of 'malic' enzymes from several sources have been determined. The rat (Magnuson *et al.*, 1986; Morioka *et al.*, 1989) and mouse (Bagchi *et al.*, 1987) sequences are highly homologous, but differ significantly from sequences of maize (Rothermel & Nelson, 1989), *Bacillus stearothermophilus* (Kobayashi *et al.*, 1989), human (Loeber *et al.*, 1991) and the dicotyledonous *C. plant* *Flaveria trinervia* (Borsch & Westhoff, 1990). The duck sequence determined in this paper is similar to those of the rat (77.3% identity) and mouse (76.8% identity). Cys-99, the sequence Val-Tyr-Thr-Pro (residues 90–93) and the nucleotide fold Gly-Leu-Gly-Asp-Leu-Gly (residues 147–152) in the duck enzyme are conserved in all of the above sequences, except for the *Bacillus* enzyme, in which Leu-151 is replaced by isoleucine.

The recombinant wild-type enzyme is kinetically indistinguishable from the natural duck enzyme, suggesting that post-translational modifications are not necessary for activity and the global structure of the protein is relatively unperturbed. The lack of structural impairment is further supported by the following lines of evidence: 1, the *E. coli*-derived enzymes are comparable with the natural duck enzyme in thermostability and in their abilities to reduce pyruvate to L-lactate; and 2, the recombinant wild-type enzyme crystallizes in the apo form, and as substrate complexes including E-NADPH, E-NADP⁺ and E-malate complexes to a maximum size of approx. $0.15 \text{ mm} \times 0.1 \text{ mm} \times 0.05 \text{ mm}$. Since the recombinant enzyme can be prepared more easily and in larger quantity than the naturally

Table 1. Purification of recombinant 'malic' enzymes

The enzymes were purified as described in the Materials and methods section. Fractions I (F-1), II (F-2) and III (F-3) represent the crude extract, the supernatant solution after heat treatment and Q-Sepharose-purified enzymes respectively. For the wild-type enzyme, 4 litres of culture was used, and for R70Q and C99S mutants, 2 litres of culture was used. The duck liver recombinant enzyme was purified by the method of Satterlee & Hsu (1991). Values in parentheses represent those obtained in additional preparations.

Enzyme	F-1 activity (units)	F-2 activity (units)	F-3		
			Activity (units)	Protein (mg)	Specific activity (units/mg)
Duck liver recombinant					30
Wild-type	2298	2122	1645	46	36 (30–45)
R70Q	1249	1293	301	11	28
C99S	508	471	347	55	6.3 (8.1)

Fig. 5. SDS/PAGE (Phastgel) of purified recombinant enzymes

The recombinant enzymes were purified as described in the Materials and methods section, heated in 2.5% SDS and electrophoresed as shown in the legend to Fig. 3. Lane 1, BSA (1 mg/ml); lanes 2 and 3, C99S at 0.3 and 0.6 mg/ml respectively; lanes 4 and 5, R70Q at 0.3 and 0.6 mg/ml respectively; lanes 6 and 7, recombinant wild-type enzyme at 0.3 and 0.6 mg/ml respectively; lane 8, duck liver 'malic' enzyme (1 mg/ml).

Fig. 6. Dependence of oxidative decarboxylase activity on substrate concentration

Kinetic assays of the natural duck enzyme were performed in 50 mM-triethanolamine buffer, pH 7.0, at 30 °C as described in the Materials and methods section. The reciprocal plots are: (a) NADP⁺ varied (5–100 μM) at fixed concentrations of MnCl₂ (100 μM) and L-malate (3 mM); (b) L-malate varied (7 μM –8 mM) at fixed concentrations of NADP⁺ (100 μM) and MnCl₂ (100 μM); (c) MnCl₂ varied (5 μM –10 mM) at fixed concentrations of NADP⁺ (100 μM) and L-malate (3 mM). K_m for NADP⁺ was obtained from (a). The K_m for L-malate, k_{cat} , and the dissociation constant of Mn²⁺ at activating metal sites (K_a) were obtained from linear segments of (b) and (c); K_a' , the dissociation constant of weakly bound metal associated with relief of L-malate inhibition, was obtained from a replot (not shown) of (c) using data at high MnCl₂ concentrations. Substrate inhibition (51 %) was calculated by a comparison of the rate at 8 mM-L-malate with k_{cat} . The rates are in $\mu\text{mol}/\text{min}$ per mg of protein and the concentrations are in μM .

Table 2. Summary of kinetic constants

The kinetic assays and calculations were performed as described in the legend to Fig. 6; 'Inhibition' refers to inhibition of k_{cat} by 8 mM-L-malate.

Enzyme	NADP ⁺ K_m (μM)	Mn ²⁺		L-Malate		
		K_a (μM)	K_a' (μM)	K_m (μM)	k_{cat} (s ⁻¹)	Inhibition (%)
Duck liver recombinant	4.6	2.4	270	73	160	51
Wild-type	5.0	2.5	290	81	169	59
R70Q	3.6	4.1	240	126	113	45
C99S	4.7	10.0	200	292	56	51

occurring enzyme, it is the preferred source for X-ray structural studies. These studies will be invaluable in the rational design of mutagenesis experiments and interpretation of data.

Arg-70 was chosen as the residue for mutagenesis on the basis of its putative role in binding the C-1 carboxyl group of L-malate (Vernon & Hsu, 1983). Replacement of this residue by glutamine removes the positive charge, and is expected to decrease (perhaps abolish) the activity on L-malate. The marginally lower catalytic efficiency of the R70Q mutant indicates, however, that Arg-70 is not the arginine residue required for L-malate binding. Further evidence is provided by the observation that susceptibility of the enzyme to phenylglyoxal is unaltered by the mutation. Arg-107 and -119 are invariant in 'malic' enzymes from animal and plant sources (Fig. 4; Magnuson *et al.*, 1986; Bagchi *et al.*, 1987; Morioka *et al.*, 1989; Rothermel & Nelson, 1989; Borsch & Westhoff, 1990; Loeber *et al.*, 1991). In future experiments, these residues need to be mutated and tested for their involvement in L-malate binding.

The decreases in k_{cat} and affinities of Mn²⁺ and L-malate at 'tight' sites observed for the C99S mutant are small but significant when viewed in terms of the relatively large decrease (27-fold) in overall catalytic efficiency accompanying the isosteric and highly conservative replacement. This result confirms our suggestion (Satterlee & Hsu, 1991) that Cys-99 is located near the malate site. Moreover, the substrate pocket is geometrically constrained, and the residue is within 'contact' distances of bound Mn²⁺ and L-malate. On the basis of results of kinetic studies, Cleland and his colleagues (Schimerlik & Cleland, 1977; Grissom & Cleland, 1988) predicted a cysteine ligand for the bound metal ion. The decreased affinity for Mn²⁺ is in accord with this prediction and suggests such a role for Cys-99. Since the serine hydroxy group may co-ordinate Mn²⁺, substitution of Cys-99 with other amino acids may be necessary to delineate the precise function of the residue in 'malic' enzyme. The decreased affinity for L-malate may be due to unfavourable orientation or electronic effects, and/or weakened metal binding since L-malate binds only to the metal (NADP⁺) complex in the ordered kinetic mechanism (Hsu *et al.*, 1967).

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REFERENCES

Bagchi, S., Wise, L. S., Brown, M. L., Bregman, D., Sul, H. S. & Rubin, C. S. (1987) *J. Biol. Chem.* **262**, 1558–1565
Borsch, D. & Westhoff, P. (1990) *FEBS Lett.* **273**, 111–115

- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Bratcher, S. C. & Hsu, R. Y. (1982) *Biochim. Biophys. Acta* **702**, 54–60
- Carter, P., Bodouelle, H., Wayne, M. M. Y. & Winter, G. (1985) Oligonucleotide Site-Directed Mutagenesis in M13, Anglian Biotechnology Ltd., Colchester
- Chang, G. G. & Hsu, R. Y. (1977a) *Biochim. Biophys. Acta* **483**, 228–235
- Chang, G. G. & Hsu, R. Y. (1977b) *Biochemistry* **16**, 311–320
- Glynias, M. J., Morris, S. M. Jr., Fantozzi, D. A., Winberry, L. K., Back, D. W., Fisch, J. E. & Goodridge, A. G. (1984) *Biochemistry* **23**, 3454–3459
- Grissom, C. B. & Cleland, W. W. (1988) *Biochemistry* **27**, 2927–2934
- Hsu, R. Y. (1982) *Mol. Cell. Biochem.* **43**, 3–26
- Hsu, R. Y. & Lardy, H. A. (1967) *J. Biol. Chem.* **242**, 520–526
- Hsu, R. Y. & Pry, T. A. (1980) *Biochemistry* **19**, 962–968
- Hsu, R. Y., Lardy, H. A. & Cleland, W. W. (1967) *J. Biol. Chem.* **242**, 5315–5322
- Hunkapiller, M. W., Granlund-Moyer, K. & Whiteley, N. W. (1986) in *Methods of Protein Microcharacterization: A Practical Handbook* (Shively, J., ed.), pp. 223–247, Humana Press, Clifton, NJ
- Kobayashi, K., Doi, S., Negoro, S., Urabe, I. & Okada, H. (1989) *J. Biol. Chem.* **264**, 3200–3205
- Loeber, G., Infante, A. A., Maurer-Fogy, I., Krystek, E. & Dworkin, M. B. (1991) *J. Biol. Chem.* **266**, 3016–3021
- Magnuson, M. A., Morioka, H., Tecce, M. F. & Nikodem, V. M. (1986) *J. Biol. Chem.* **261**, 1183–1186
- Morioka, H., Magnuson, M. A., Mitsuhashi, T., Song, M. K. H., Rall, J. E. & Nikodem, V. M. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 4912–4916
- Nevaldine, B. H., Bassel, A. R. & Hsu, R. Y. (1974) *Biochim. Biophys. Acta* **336**, 283–293
- Pry, T. A. & Hsu, R. Y. (1978) *Biochemistry* **17**, 4024–4029
- Pry, T. A. & Hsu, R. Y. (1980) *Biochemistry* **19**, 951–962
- Rothermel, B. A. & Nelson, T. (1989) *J. Biol. Chem.* **264**, 19587–19592
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning*, 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
- Sanger, F., Nicklen, D. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463–5467
- Satterlee, J. & Hsu, R. Y. (1991) *Biochim. Biophys. Acta* **1079**, 247–252
- Schimerlik, M. I. & Cleland, W. W. (1977) *Biochemistry* **16**, 576–582
- Tang, C. L. & Hsu, R. Y. (1973) *Biochem. J.* **135**, 287–291
- Tang, C. L. & Hsu, R. Y. (1974) *J. Biol. Chem.* **249**, 3916–3922
- Vernon, C. M. & Hsu, R. Y. (1983) *Arch. Biochem. Biophys.* **225**, 296–305
- Winter, G., Fersht, A. R., Wilkinson, A. J., Zoller, M. & Smith, M. (1982) *Nature (London)* **299**, 756–758

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