Duck liver ‘malic’ enzyme

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

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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...
obtained from commercial sources. Restriction endonucleases, T4 DNA ligase, polynucleotide kinase and DNA polymerase I (Klenow fragment) were purchased from Boehringer Corp., and used according to the manufacturer’s instructions. [γ-32P]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 μmol 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 Psrl site of pBR322 (Glynnias et al., 1984). It was digested with Psrl 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 x YT agar plates with 5-bromo-4-chloro-3-indolyl-β-D-galactoside (0.1 mM), isopropyl β-D-thiogalactopyranoside (IPTG; 0.15 mM) and ampicillin (100 μg/ml). Individual β-galactosidase-negative colonies containing the insert were grown overnight in 2 x YT (100 μg 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 Psrl 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 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'-TCGAAATGAAAGCT-CTTCTAT-3'; 5'-TCGGCTGCTATGGCATGGA-3'; 5'-TTGCTAATGCCAATTCGCGG-3'; 5'-AAATATGGATTGTGACTCAAAAG-3'; 5'-AGCAATCTACTCGAAAAG-3'; and 5'-GTCGCTGACCCCTCTTTAGT-3'. Those used to sequence the reverse strand were: universal; 5'-TGTAGGCTTCTCCTGAGTC-3'; 5'-TGCTTTGTCTGGTACGATGGATTGC-3'; 5'-CCATAGCCATGACAATGAGG-3'; 5'-GATATTCTTTTGCTTCTCAGGC-3'; and 5'-GACCGAACGGTGTATTTAT-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 BamHI and HindIII 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 Psrl fragment containing ‘malic’ enzyme cDNA insert. In pHF-1, the Psrl fragment (DME) and ampicillin-resistance gene (AMP) are both cross-hatched.
Malic enzyme expression

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 PstI 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 T4 DNA ligase. The resultant product was used to transform competent E. coli BMH 71-18 mutL cells by the CaCl₂ method. Colonies grown from overnight cultures were then subjected to screening with γ-[^32]P-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'-TTCAGGATCAGAATGAAAAG-3'). The active-site cysteine was identified as residue 99 (Satterlee & Hsu, 1991) by labelling the enzyme with 3-bromo-[1-^14]Cpyruvate, and aligning the sequence of the chromatographically purified radioactive trypsic peptide with that of the natural duck enzyme. [7-^14]CPhenylglyoxal 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-Ultrasones 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% (v/v) satd. (NH₄)₂SO₄, 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 PstI 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 EcoRI 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
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 μl 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 μM-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 μM) 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 μM-MnCl₂ and 3 mm-l-malate (Fig. 6a) was linear and yielded a Kₘ of 4.6 μM for the nucleotide. L-Malate and Mn²⁺ each gave biphasic plots, and required the use of broad concentration ranges (7 μM–8 mm for L-malate and 5 μM–10 mm for MnCl₂) for proper definition. The plot in which L-malate was varied (Fig. 6b) at 100 μM-MnCl₂ and saturating (100 μM) NADP⁺ was linear from 7–60 μM, but curved upwards with higher concentrations (> 360 μM) because of
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[ACG ACG ATT ACG ATT TCC CCG GGA CTC GTC GAC CTC CAG ACC]
[Thr Met Ile Thr Aan Ser Arg Gly Ser Val Aasp Leu Gin Leu Thr]

1  ATG AAG AAG GCC TAC GAG GGG CTC CCG GAC CCT CTC AAC AAG GGA ATG GCA ATT ACC  60
   Met Lys Arg Gly Tyr Glu Val Leu Arg Aasp Pro His Leu Aan Lys Gly Met Ala Phe Thr  20
51  TTG GAG GAG AGG CAA GGA CTT AAT ATT CAT GAA CTA TGG CCA CCT GTC ATT CAA  120
   Leu Glu Glu Arg Gin Gin Leu Aan Aaa His Glu Leu Pro Pro Cys Phe Leu Gly Gin  40
121  GAT GTC GCT GTG TTC ATT ATT CAG AAG ATT TTT CAA GAA CGA ACA TCC GCA GAC AGA  180
   Aasp Val Gin Glu Val Phe Arg Arg Arg Pro His Arg Thr Ser Aasp Arg Arg Arg  60
181  TAC ATT CTA CTA ATT AAT GTC GAT CAA GGT ATT AAT AAT ATT AAT AAT ATT AAT  240
   Tyr Ile Leu Met Leu Met Gin Gin Arg Arg Gin Gin Arg Gin Gin Gin Gin Gin Gin  80
241  TCT GAC AYA GAA GAG TTC AYG CCT ATT GYT TAT ACT CCT ACT CTG GCA CTC GCT CAG  300
   Ser Arg Ile Glu Arg Met Pro Ile Val Thr Tyr Thr Thr Thr Thr Thr Thr Thr Thr Thr 100
301  CAA TAT GTG TTA GCA TTT CAG AGG CAA GGA CTT TTT ATT ACT ACT CAG CTA GCG CGA  360
   His Tyr Glu Ala Phe Arg Arg Arg Pro Arg Gly Leu Phe Ile Thr His Arg Arg Arg 120
361  CAC ATG CCT GCA AAG CCT TGC CCA GAA AAT GTC ATC AAG AAT ATT GGT GGT ACA  420
   His Ile Thr Met Leu Lys Ser Thr Pro Gin Ser Val Ile Lys Ala Val Val Thr  140
421  GAT GGA CAA ATT ATT GTT CCT GTC GAC CTC GTC TAT GGG AGC ATC CCG GGT  480
   Aasp Gly Arg Arg Arg Arg Arg Pro Thr Pro Asp Thr Thr Thr Thr Pro Thr Thr Thr  160
481  GGT AAA CTT GCA GCT TAT ACC CCG TCT CCT CCT GCC GCA GGT AGA AAT TCT CCA GG  540
   Gly Lys Ala Ala Thr Ala Ala Thr Ala Thr Ala Thr Ala Thr Ala Thr Ala Thr Ala  180
541  ATG TTA GGT GGA ACA GAT ATT GAG CCA CTG CTG AAA GAT CTT TTA ATT GCT CTT  600
   Met Leu Aasp Val Gly Arg Thr Aasp Gin Gin Leu Leu Leu Leu Leu Leu Leu Leu Leu Thr Leu  200
601  AAG CAC AGA AGA AAT GCT CTA TAT GCC CAG GAG GTG CAG GCC CTA GCC GCA CTC GAG  660
   Arg His Arg Arg Arg Arg Asp Gin Phe Thr Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin  220
661  GGT ACT TCA AAG TAT GGC AAC ACC TCC ATT CAA AAG AAC TAT GCT CTT GAT  720
   Val Thr Ser Arg Arg Gin Gin Gin Arg Gin Gin Thr Gin Thr Thr Gin Thr Thr Gin  240
721  GCA TTC CCC CTC CTT GAT CAT AAG TCT CCT AAG ATT TAT ACT TAT AAT GAT GAC ATT CAA  780
   Ala Phe Arg Arg Phe Arg Arg Arg Arg Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr  260
781  GCA ACT GCA TCT GTC GCT GTC GCT GCT GCT GCT GCT ACT ACT AAG AAG  840
   Thr Thr Ala Ser Val Val Val Val Val Val Val Val Val Val Val Val Val Val Val  280
841  GTA TCA GAT CAC ACA GGT CTC GTG GTG GCC CCT GCC GTA GCC GTA GTA GAA CAC  900
   Met Thr Arg Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin  300
891  CTC ATT GGC GTG GCT AGT CGC AAC AAT CGA TCT ATT CGG CCA GAA CGA CTG CGA GAT GCT  960
   His Thr Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin  320
961  GGT ACT TGC CCT ATT ACC AAG TCA TTT AAC GGT CTT GCT TCA AGT CAA CAC GAG AAA  1020
   Val Thr Ser Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr  360
1021  ACC CAA TTT GCA CAT GAA CTT GCT GGA ATT AGA AAT CTA GTA GAT ATT GGT AAA AAT  1080
   Thr Arg Thr Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin  360
1081  AAT TAT TAT ATT ATT ATT ATT TTG GCT TAT ACC AAT AAG TAT ATT GCT ATT  1140
   Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr  380
1141  CAG GAT ATT CRT CRT CTC AAG AAA ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT  1200
   Gin Aasp Arg Arg Arg Arg Arg Arg Arg Arg Arg Arg Arg Arg Arg Arg Arg Arg Arg Arg  400
1201  AAA GCA GAC TCC TCT GTC TCC AAG ATT TAT GGT CAT GCA TCC TCT TCA GTC GAT GAA  1260
   Arg Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin  420
1261  GAT GGA ATT TTT TAT CCT GTC ACT ATT CCA AAG GAA ATT GGT ATT ATT ATT ATT ATT  1320
   Gin Aasp Ser Pro Pro Aasp Met Pro Val Pro Pro Pro Val Pro Pro Pro Val Pro Pro  440
1321  GCT AAC AAT TCT TAT GGT TCC CCC TCA GAT CTT AGT ATT ATT ATT ATT ATT ATT ATT  1380
   Gin Ser Ser Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr  460
1381  GAT GCT TAC AAG GGT TTT TCC ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT  1440
   Gin Thr Thr Aasp Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr  480
1441  GAT GGC CTC GGT CTT GCT CTC TCT CTT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT  1500
   Gin Aasp Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr 500
1501  GGT GAT GGA ATT TAT TAT TAT GGC ACC ATT TAC AAG GGA GAT ATT ATT ATT ATT ATT  1560
   Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr 520
1561  GCT GCA AGA ATT TCT TAC AAG GAT TAT AAC ATT TCT TCT CTC AAG AAT ATT TCT CTA  1620
   Gin Thr Gin Aasp Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr  540
1621  TTC GTC GCT GCA TAT ACC TCT GGT CAT GAA GGA CCG ATT AAA CGG AAA TCT TAA  1674
   Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr  557

541  Phe Val Ala Aasp Ser Tyr Thr Thr Pro Val Pro Glu Glu Ala Met Lys Val Lys Leu ***
substrate inhibition. This inhibition was relieved by MnCl₂ at concentrations greater than 150 μM, 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 MnCl₂ 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 MnCl₂ 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_cat/(K_m NADP⁺ × K_m L-malate × 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., 1993) and the dicotyledonous C₄ 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 mm × 0.1 mm × 0.05 mm. Since the recombinant enzyme can be prepared more easily and in larger quantity than the naturally

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**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.

<table>
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<th>Enzyme</th>
<th>F-1 activity (units)</th>
<th>F-2 activity (units)</th>
<th>Activity (units)</th>
<th>Protein (mg)</th>
<th>Specific activity (units/mg)</th>
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<tr>
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<td>471</td>
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**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 MnCl\(_2\) (100 μM) and L-malate (3 mM); (b) L-malate varied (7 μM–8 mM) at fixed concentrations of NADP\(^+\) (100 μM) and MnCl\(_2\) (100 μM); (c) MnCl\(_2\) varied (5 μM–10 mM) at fixed concentrations of NADP\(^+\) (100 μM) and L-malate (3 mM). \(K_a\) for NADP\(^+\) was obtained from (a). The \(K_a\) for L-malate, \(k_{cat}\), and the dissociation constant of Mn\(^{2+}\) 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 MnCl\(_2\) concentrations. Substrate inhibition (51 %) was calculated by a comparison of the rate at 8 mM-L-malate with \(k_{cat}\). The rates are in μmol/min per mg of protein and the concentrations are in μM.

Table 2. Summary of kinetic constants

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>NADP(^+)</th>
<th>Mn(^{2+})</th>
<th>L-Malate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(K_m) (μM)</td>
<td>(K_a) (μM)</td>
<td>(K'_a) (μM)</td>
</tr>
<tr>
<td>Duck liver recombinant</td>
<td>4.6</td>
<td>2.4</td>
<td>270</td>
</tr>
<tr>
<td>Wild-type</td>
<td>5.0</td>
<td>2.5</td>
<td>290</td>
</tr>
<tr>
<td>R70Q</td>
<td>3.6</td>
<td>4.1</td>
<td>240</td>
</tr>
<tr>
<td>C99S</td>
<td>4.7</td>
<td>10.0</td>
<td>200</td>
</tr>
</tbody>
</table>

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; Loeb 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 Mn\(^{2+}\) 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 Mn\(^{2+}\) 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 Mn\(^{2+}\) is in accord with this prediction and suggests such a role for Cys-99. Since the serine hydroxy group may co-ordinate Mn\(^{2+}\), 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).

This work was supported by grants from U.S. National Institutes of Health DK-13390 (R. Y. H.) and DK-21594 (A. G. G.), American Heart Association New York Chapter (R. Y. H.) and British Science and Engineering Council GR/F7398.4 (J. J. H. and R. Y. H.).

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Received 20 August 1991/30 October 1991; accepted 15 November 1991