Preparation and Kinetic Characterization of a Fusion Protein of Yeast Mitochondrial Citrate Synthase and Malate Dehydrogenase†

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ABSTRACT: We have expressed the DNA of the fusion of CS1 to MDH1 in *Escherichia coli* gltA⁻. The fusion protein (CS1/MDH1) is the C-terminus of CS1 linked in-frame to the N-terminus of MDH1 with a short linker of glycyl-seryl-glycyl. The fusion protein produced was isolated and purified. Gel filtration studies indicated that CS1/MDH1 had a M_r of ~170 000. Western blotting analysis with SDS gel indicated a M_r of ~90 000–95 000 (theoretical M_r = 87 000). This is the expected M_r for the fusion protein subunit. The kinetics of CS1 and MDH1 activities of the fusion protein were compared to those of the free enzymes. In addition, the effect of AAT reaction, as a competitor for the intermediate OAA of the coupled MDH–CS reaction, was examined. It was observed that AAT was a less effective competitor for OAA when the CS1/MDH1 fusion protein is used than when the separate enzymes are employed. In addition, the transient time for the coupled reaction sequence was less for the fusion protein than for the free enzymes.

Over the last decade it has been demonstrated that many enzymes that act within a metabolic pathway interact with each other to form organized enzyme complexes (Srere, 1987). A considerable body of evidence has been presented which demonstrates these interactions and shows differences in kinetics of enzymes which catalyze sequential metabolic reactions when they are in close proximity compared to the kinetics of the same enzymes in random solution (Bülow, 1987; Ljungcrantz *et al.*, 1989).

Among the mitochondrial enzymes of the Krebs TCA[1] cycle, interactions between sequential enzymes have been demonstrated between six of eight possible interactions (Srere, 1985). Also, high molecular weight complexes of the Krebs TCA enzymes have been isolated (Barnes & Weitzman, 1986), and kinetic effects have been observed in some sequential reactions within this complex compared to corresponding free enzymes (Robinson *et al.*, 1987; Sumegi *et al.*, 1992).

A problem in demonstrating the *in situ* existence of enzyme complexes as well as demonstrating possible advantages of these complexes is that during isolation many of them tend to dissociate due to dilution effects. A number of model systems have therefore been used to investigate the effects of close proximity of sequentially operating enzymes. For instance, the enzymes CS and MDH have been covalently coupled to a Sepharose matrix and the kinetics of the sequential reaction investigated (Srere *et al.*, 1973). The results showed a reduction in the lag time of the overall enzyme reaction and a higher steady-state rate compared to a solution of the free enzymes. To study the possible effects of the immobilizing matrix material on the enzymes, they were also chemically cross-linked to each other in solution, yielding soluble enzyme conjugates (Koch-Schmidt *et al.*, 1977). However, these conjugates did not exhibit any kinetic advantages compared to a system with free enzymes except when precipitated with PEG. This may have been due to effects caused by the cross-linking method which in general is difficult to control. In another study, Spivey and co-workers were able to demonstrate intermediate substrate (OAA) sequestration in the overall reaction of malate to citrate using a PEG coprecipitated solid-state preparation of pig CS and MDH (Datta *et al.*, 1985).

It has been proposed that a better model for the study of proximity effects would be to use a system consisting of sequential enzymes fused by molecular biological techniques (Bülow & Mosbach, 1991). A number of bienzymatic fusion proteins which catalyze sequential reactions have been constructed (Bülow, 1987; Ljungcrantz *et al.*, 1989). They have shown kinetic benefits for the overall reaction, such as shorter lag times (transient times) (Bülow, 1987; Ljungcrantz *et al.*, 1989) and an apparent sequestering of the intermediate by the fusion system when compared to the free enzymes in the presence of an enzyme trap for the intermediate metabolite (Bülow, 1987).

In this paper we describe the construction of a bifunctional enzyme conjugate consisting of CS1 genetically fused, with a short linker, in-frame with MDH1. The conjugate (CS1/MDH1) is expressed in *Escherichia coli*, isolated, partially characterized, and used to study the overall sequential reaction of malate to citrate. The results are consistent with other observations on fusion proteins in that the intermediate substrate, OAA, is apparently more sequestered by the fusion protein than in the system with free enzymes, as judged by its availability to an added scavenger enzyme, AAT.

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[1] Abbreviations: CS, citrate synthase; CS1, yeast mitochondrial citrate synthase; MDH, malate dehydrogenase; MDH1, yeast mitochondrial malate dehydrogenase; TCA, tricarboxylic acid; AAT, aspartate aminotransferase; PEG, poly(ethylene glycol); OAA, oxalacetate; IPTG, isopropyl β-D-thiogalactoside; SDS, sodium dodecyl sulfate; DTNB, dithionitrobenzoate; BSA, bovine serum albumin.

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EXPERIMENTAL PROCEDURES

Materials. Restriction enzyme endonucleases, T4 DNA ligase, Taq DNA polymerase, and IPTG were supplied by Boehringer Mannheim (Mannheim, Germany). Primers 5′-CCATGGGAGCTCGCCTCCGAACAAACGTTG-AAGGAGAGATTTGC-3′ and 5′-GACATCCTGCAGC-TATTTACTAGCAACAAAGTTGAC-3′ for PCR amplification of the fusion protein, CS1/MDH1, were synthesized on a 381-A DNA synthesizer (Applied Biosystems, Foster City, CA) using the phosphoramidite method at the Molecular Biology Core Facility at the University of Lund (Lund, Sweden).

Ammonium sulfate suspensions of pig CS (EC 4.1.3.7) and AAT (EC 2.6.1.1) were from Sigma Chemical Co. (St. Louis, MO). NADH, malate, OAA, DTNB, and glutamic acid were also from Sigma. CS1 was prepared in this laboratory (Brent & Srere, 1987); some MDH1 was a generous gift of Dr. Lee McAlister-Henn (University of Texas Health Science Center, San Antonio, TX), and some was also prepared by us.

All restriction enzyme reactions were carried out as recommended by the manufacturer. DEAE-Sepharose was obtained from Pharmacia-LKB (Sollentuna, Sweden). All other chemicals used were of the highest purity available.

Bacterial Strains and Plasmids. E. coli DEK15 (CS^-) and *E. coli* (MDH^-) was used for standard transformation procedures (Maniatis *et al.*, 1982). The bacterial expression plasmid pTrc99A was purchased from Pharmacia-LKB. Plasmid pCS1/MDH1, expressing the fusion protein CS1/MDH1, is a yeast expression vector and has been described elsewhere (Lindbladh *et al.*, 1994). Bacterial cells were grown and selected using standard methods, and IPTG and ampicillin were included at a final concentration of 5 mM and 100 μg/mL when necessary.

Production and Purification of the CS1/MDH1 Fusion Protein. The bacterial culture was grown in Luria–Bertani medium containing ampicillin at 37 °C for 24 h in the presence of 5 mM IPTG. After centrifugation at 6000*g* for 10 min the cell pellet was washed with 50 mM Tris-HCl and 1 mM EDTA (pH 7.5). The cells were frozen by dripping them into liquid nitrogen and stored at −70 °C.

For enzyme isolation, 20 g of frozen cells was thawed in 10 mM sodium phosphate buffer (pH 7.4), containing 1 mM EDTA, 1 mM β-mercaptoethanol, 1 mM PMSF, and 1 mM benzamidine (9 g of wet cells/10 mL), and frozen again into liquid nitrogn and thawed at room temperature. The cell suspension was lysed by addition of lysozyme (0.2 mg/mL suspension) for 30 min at room temperature on a rocking table and sonicated at 0 °C by alternating four cycles of 30-s sonication with 1-min intervals. All subsequent operations were carried out at 4 °C. The lysate was cleared by centrifugation at 12000*g* for 30 min. Nucleic acids were removed from the supernatant solution by precipitation with 5 mg of protamine sulfate/g of wet cells and centrifugation for 10 min at 20000*g*. A 50% (w/v) solution of PEG (M_r = 4000) (in the sodium phosphate buffer described above) was added slowly to the supernatant solution previously obtained until 33% saturation of PEG. For every 5% added, the sample was incubated for 15 min at 10 °C and centrifuged for 10 min at 20000*g*, and the supernatant was checked for CS activity. The corresponding pellets which do not contain any CS activity were discarded. After 33% saturation of PEG, all the CS activity was recovered into the pellet which was resuspended in a small volume of 10 mM sodium phosphate, 1 mM EDTA, and 1 mM β-mercaptoethanol (pH 7.4) and loaded onto a 10-mL Blue-Sepharose column previously equilibrated with the same buffer. The column was washed with 100 mL of the sodium phosphate buffer (pH 7.4), and bound proteins were eluted with a 120-mL gradient of 0.0–0.5 M KCl. The fractions containing both CS and MDH activities were pooled and precipitated with the addition of 70% solid ammonium sulfate. The pellet was resuspended in 50 mM Tris-HCl (pH 7.5), dialyzed overnight against the same buffer, and loaded onto a 15-mL DEAE-Sepharose column previously equilibrated with the same buffer. The column was washed with 75 mL of 50 mM Tris-HCl (pH 7.5), and bound proteins were eluted with a 150-mL, 0.0–0.2 M NaCl gradient. The fractions containing both CS and MDH activities were pooled, concentrated, and kept in 50 mM Tris-HCl (pH 7.5) and 50% glycerol at −70 °C.

Enzyme Assays. CS activity was determined using 0.2 mM acetyl-CoA, 0.5 mM OAA, and 1 mM DTNB in 100 mM Tris-HCl (pH 7.5). The reaction was followed spectrophotometrically at 412 nm (Srere *et al.*, 1963). MDH was assayed using 0.1 mM NADH and 0.1 mM OAA in 50 mM Tris-HCl (pH 7.5) at 340 nm (Englard & Siegel, 1969). AAT was assayed with 30 mM aspartate, 2.5 mM α-ketoglutarate, 0.05 mM pyridoxal phosphate, 0.4 mM DTNB, 0.1 mM acetyl-CoA, and excess pig CS in 100 mM potassium phosphate buffer (pH 7.5) at 412 nm. Extinction coefficients were assumed to be 13 600 and 6200 M^{-1} cm^{-1} for DTNB and NADH, respectively. One unit of enzyme liberates 1 μmol of product/min. All reactions were measured at room temperature.

Coupled Enzyme Reaction. The overall reaction of malate to citrate catalyzed by MDH and CS, either free or fused, was monitored in 10 mM malate, 4 mM NAD, and 0.1 mM acetyl-CoA using 0.4 mM DTNB at 412 nm in 40 mM potassium phosphate buffer (pH 8.1). A scavenger system for the intermediate OAA, AAT, and 4 mM glutamate was added to the reaction mixture at different concentrations of AAT to measure any differences between the system with free CS and MDH and that with equivalent enzyme activities of the fused CS1/MDH1. Enzyme activities used are further specified in the text. All values are mean values obtained from triplicate analysis.

Electrophoresis and Western Blotting. SDS–PAGE was performed on a 10% polyacrylamide slab gel using the Tris-HCl (pH 8.3) discontinuous buffer system according to Laemmli (1970). The M_r of the fusion protein was determined by comparing the relative mobility with those of the standard calibration proteins obtained from Pharmacia-LKB. Western blotting was performed as recommended by the manufacturer, JKB-Biotech (Denmark). Antibodies used in the reaction were anti-CS1 rabbit immunoglobulin, anti-MDH1 rabbit immunoglobulin prepared by us, and peroxidase-conjugated anti-immunoglobulin (Dakapats). H_2O_2 and 4-chloro-1-naphthol were employed as substrates to visualize the blotted bands. Protein concentrations were determined according to Bradford (1976) using BSA as the standard.

N-Terminal Sequence Analysis. Components of the protein mixture were separated for amino acid sequence analysis using the method of Matsudaira (1987). A sample containing 13.5 μg of protein was subjected to SDS electrophoresis in a 10% polyacrylamide gel and electroblotted to an Immobilon SQ membrane (Millipore Corp., Bedford, MA), and the components were located by staining the blot with Coomassie Blue R-250. Bands determined by staining intensity to contain 1.5 μg (50 pmol) of the 30-kDa species, 2.0 μg (40 pmol) of the 50-kDa species, and 10 μg (120 pmol) of the 85-kDa species

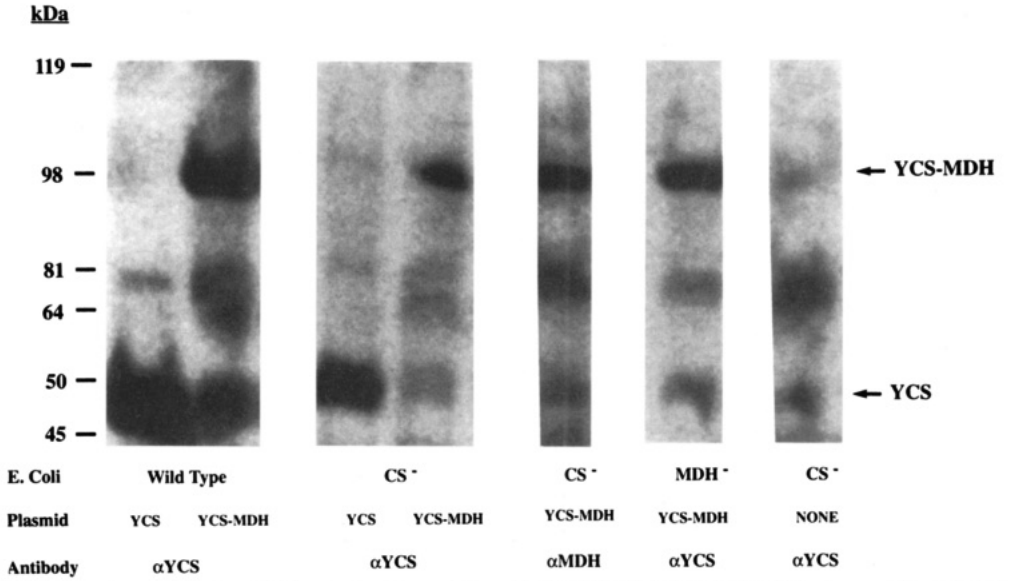

FIGURE 1: Gel electrophoresis and Western blot analysis of recombinant CS1/MDH1 (YCS–MDH) fusion proteins in MDH and CS mutants and parental *E. coli*. Cells were grown as described in Experimental Procedures. Total cell protein lysates were prepared as described in Experimental Procedures, and 20 μg was separated by 10% SDS–PAGE. Molecular weight standards and authentic CS1 and MDH1 standards were run as controls. Arrows denote the position of immunoreactive CS1 (YCS) and CS1/MDH1 (YCS–MDH) fusion proteins.

were excised and submitted to automated Edman degradation using a model 477A sequencer from Applied Biosystems Inc. (Foster City, CA). Phenylthiohydantoin amino acid derivatives were identified using an on-line model 120A PTH amino acid analyzer.

Protein Immunoblot Analysis. MDH and CS mutants and parental *E. coli* cells were grown in 1.0 L of Luria broth, and the synthesis of the CS1/MDH1 fusion proteins was induced by 5.0 mM IPTG for 24 h at 37 °C. Cells were centrifuged at 10000*g* at 4 °C for 20 min, washed once with 1 × M9 medium, centrifuged at 4500*g* at 10 °C for 20 min, and resuspended in 1.0 mL of 1 × M9 medium. Total cell protein lysates were prepared by freeze/thaw lysis on dry ice/ethanol twice and centrifugation of cell debris at 14000*g* for 10 min at 4 °C. Total proteins, 20 μg, were separated by 10% SDS–PAGE. The samples were electrotransferred onto a nitrocellulose membrane and developed with a 10^{-4} dilution of a rabbit polyclonal α-YCS serum (no. 700) or a rabbit polyclonal α-MDH serum (no. 276) and 10 μCi of ^{125}I-labeled protein A. The filters were blocked for 1 h at room temperature in 100 mL of PBS containing 0.05% Tween 20, 0.5 M NaCl, and 1% BSA (buffer A) and incubated overnight at room temperature in the same solution containing the primary antibodies. Filters were washed twice for 20 min in buffer A at room temperature and then once for 20 min in PBS containing 0.5 M NaCl and 1% BSA (buffer B). The blots were incubated for 2 h at room temperature for 100 mL of buffer B containing 10 μCi of ^{125}I-labeled protein A (specific activity 1110 MBq/mg) and washed twice for 10 min in buffer B and finally washed for 10 min in PBS. Immunoreactions were developed by autoradiography overnight at –80 °C with intensifying screens (Du Pont). Molecular weight standards and authentic CS and MDH standards were run as controls.

Determination of Molecular Weight by Column Chromatography. A Sephacryl HR300 column (1 × 100 cm) was used. The column was equilibrated with 50 mM Tris-HCl (pH 8.0) and 100 mM KCl. Elution was with the same buffer, and 0.7-mL samples were collected and analyzed for CS and MDH activities.

RESULTS

CS1/MDH1 Fusion Protein. The plasmid pCS1/MDH1 encoding the fusion protein CS1/MDH1 for expression in yeast mitochondria has been described before (Lindbladh *et al.* 1994). To obtain an expression vector for use in *E. coli*, the gene encoding the fusion protein was amplified using PCR, digested with *Pst*I and *Sac*I and inserted into plasmid pTrc99A previously digested with *Pst*I and *Sac*I. The resulting plasmid has an IPTG-inducible trc promoter and encodes Met-Glu-Phe-Glu-Leu followed by the entire CS1 fused in-frame with a short linker to the N-terminus of the entire MDH1. The linker region between the two enzymes consists of three amino acids, Gly-Ser-Gly.[2] The individual nucleic acid sequences of CS1 and MDH1 have been described before (Suissa *et al.*, 1984; Thompson *et al.*, 1988).

Several strains of *E. coli* have been transformed with the plasmid pCS1/MDH1. These were in parental strain, a gltA$^-$ (CS$^-$) strain, and an MDH$^-$ strain. The latter strains cannot grow on acetate. In the case of the gltA$^-$ and MDH$^-$ strains, growth on acetate was restored by the transformation with the plasmid. In addition, Western blotting after SDS–PAGE of extracts of these transformed bacteria showed the production of a protein which reacted with both anti-MDH1 and anti-CS1 with an apparent M_r of 90 000–95 000 (Figure 1). In addition, we sometimes observed a protein ($M_r \sim$ 80 000) which reacts with both anti-MDH and anti-CS. This is probably a proteolytic product of the fusion protein.

Characterization of the CS1/MDH1 Fusion Protein. The hybrid enzyme could be produced and purified according to the protocol described in Experimental Procedures (Table 1). The profile of proteins at each stage of purification is illustrated in Figure 2. Upon PEG electrophoresis in the presence of SDS the purified sample of the fusion protein gave three major bands: one corresponding to an apparent molecular mass of ~85 kDa, the second to a molecular mass of ~50 kDa, and the third to a molecular mass of ~30 kDa. The N-terminal

[2] The original construct for the fusion protein was with a Gly-Ser-Gly linker. It is probable that a point mutation occurred during its expression in *E. coli* to yield a Gly-Ser-Ala linker.

Table 1: Purification of the Fusion Protein from *E. coli* DEK15 (CS^-)[a]

steps	proteins (mg)	CS1 activity (units)	SA_{CS1} (units/mg)	MDH1 activity (units)	SA_{MDH1} (units/mg)	SA_{MDH1}/SA_{CS1} ratio
sonication	2020	2510	1.24	21750	10	8.1
protamine sulfate	1990	1880	0.94	22500	11	11.7
20% PEG	630	1630	2.6	3300	5.2	2.0
Blue-Sepharose	8.53	270	31.6	880	103	3.2
DEAE-Sepharose	2.25	136	60.4	190	84.9	1.4

[a] The results given in this table come from the purification of 20 g of frozen cells as described in Experimental Procedures. The MDH activity was measured from OAA to the formation of malate. SA, specific activity.

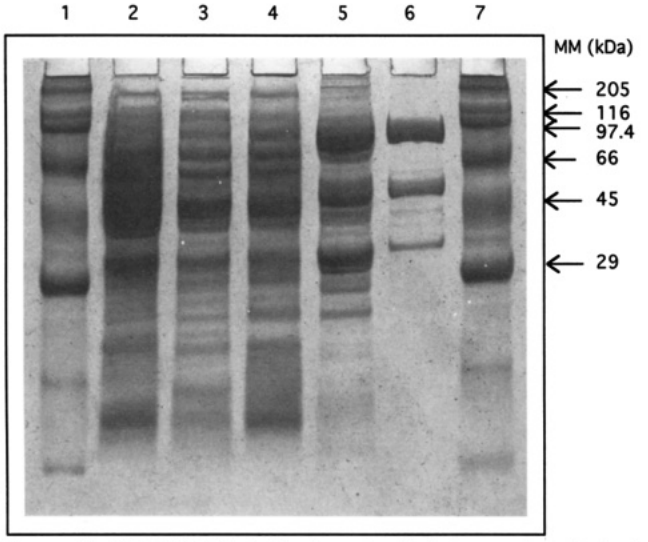

FIGURE 2: SDS–PAGE electrophoresis. Protein samples obtained after each step in the purification procedure were electrophoresed on SDS–polyacrylamide gel and stained with Coomassie Blue. Lanes: 1 and 7, 10 μg of standard proteins; 2, 14 μg of crude extract obtained after sonication of the sample; 3, 14 μg of sample obtained after protamine sulfate precipitation; 4, 14 μg of proteins loaded on a Blue-Sepharose column; 5, 8 μg of proteins loaded on a DEAE-Sepharose column; 6, 4 μg of the purified CS1/MDH1 fusion protein.

sequence analysis of the 85-kDa protein was that of CS1 and that of the 50 kDa was also CS1, while that of the ~30-kDa band was the last five carboxy-terminal amino acids of CS1, followed by the linker of Gly-Ser-Ala,[2] followed by the N-terminus of MDH1. They correspond respectively to the association between one subunit of CS and one subunit of MDH (85 kDa), to one subunit of CS (50 kDa), and to one subunit of MDH preceded by the tripeptide used for the fusion and the last five amino acids C-terminal of CS. When the fusion protein is examined by gel filtration chromatography, then overlapping CS1 and MDH1 activities are found at a molecular mass of ~167 000 daltons as expected (Figure 3). Since the M_r of CS is 100 000 and that of MDH is 67 000, then a simple fusion protein would be expected to have a mass of 167 000 daltons. We do see some overlapping CS1 and MDH1 activities eluting at a position of high molecular weight. These latter data would indicate therefore that some higher order aggregate of the fusion protein may have been formed.

Kinetics of CS1 and MDH1 in the Fusion Protein. The K_ms for acetyl-CoA and OAA in the CS1 forward reaction were measured. For acetyl-CoA an increase from 5.6 ± 0.3 μM for the free enzyme to 7.6 ± 0.1 μM for the CS1 in the fusion protein and an increase for K_m for OAA from 5 ± 0.4 μM to 7.50 ± 0.14 μM were found (Table 2).

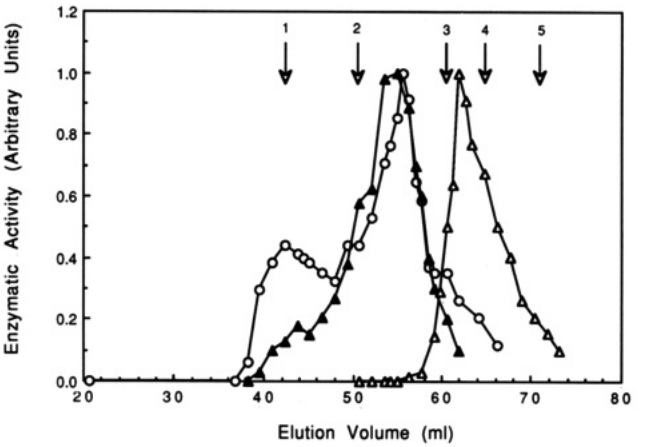

FIGURE 3: Gel filtration chromatography on Sephacryl S300 of the CS/MDH fusion protein. The column (1.0 × 100 cm) was equilibrated with 50 mM Tris-HCl buffer (pH 8.0) and 100 mM KCl. The first 20 mL was collected separately. Curves: (○) CS activity of the fusion protein; (▲) MDH activity of the fusion protein; (△) *E. coli* MDH activity. The arrows indicate the elution position of different proteins used as molecular weight standards: (1) ferritin, 450 000; (2) catalase; 232 000; (3) pig CS, 100 000; (4) pig MDH, 70 000; and (5) ovalbumin, 45 000.

Table 2: Kinetic Constants for the Free Enzymes and the Fusion Protein

	V_m (μmol of product/min)	K_m(AcCoA) (μM)	K_m(OAA) (μM)
CS1	38 ± 2	5.6 ± 0.3	5 ± 0.4
CS1/MDH1	28 ± 2	7.6 ± 0.1	7.5 ± 0.14
	V_m (μmol of product/min)	K_m(OAA) (μM)	K_m(NADH) (μM)
MDH1	83 ± 4	23 ± 3	30 ± 2
MDH1 in CS1/MDH1	69 ± 8	9.4 ± 1.2	13.6 ± 1.5
	V_m (μmol of product/min)	K_m(malate) (mM)	K_m(NAD) (mM)
MDH1	20 ± 2.5	0.28 ± 0.02	0.3 ± 0.08
MDH1 in CS1/MDH1	24 ± 2.5	0.14 ± 0.01	0.11 ± 0.02

In the case of MDH about a 50% decrease for MDH in the fusion protein in the K_m values for OAA, NADH, malate, and NAD was observed. In addition, no difference between the conjugate MDH and free MDH in substrate inhibition by OAA was noted for the MDH reaction in the OAA to malate direction. We have checked the possibility that the presence of both CS1 and MDH1 in the reaction mixture could affect the K_m values. When the K_ms for OAA and NADH in the MDH1 reaction are determined in the presence of CS1, the same results are obtained as those obtained when the experiment is carried out with no CS1. Also, when the K_ms for OAA and acetyl-CoA in the CS1 reaction are determined in the presence of MDH1, the same results are obtained as that with CS1 alone. So the differences in the K_m values between the native enzymes and the fusion protein reflect the presence of specific interactions resulting from the fusion of the enzymes.

Coupled Reaction. The individual enzyme activities for the fused and free enzyme systems were matched to form systems with equal V_{max} values for CS1 and MDH1. Different amounts of an OAA-trapping enzyme, AAT, were added to the reaction mixture, and the overall reaction was monitored using DTNB as described in Experimental Procedures (Figure 4A). The system consisting of the fusion protein, CS1/MDH1, is less affected compared to the system with free enzymes when the OAA-trapping enzyme, AAT, is included. The overall reaction for the system based on the fusion protein

Table 3: Effect of Malate on the Transient Time of the Overall Reaction for the Fusion Protein or the Native Enzymes[a]

enzymes	malate (mM)		
	7	0.9	0.09
fusion protein (FP)	4.4 ± 1.0 (4.4 ● 1)[b]	4.8 ± 0.7 (4.4 ± 1)	4.3 ± 0.5 (4.4 ± 1)
native enzymes (NE)	7.0 ± 1.5 (7.0 ● 1.5)	9.5 ± 0.5 (7.0 ± 1.5)	10 ± 1.0 (7.0 ± 1.5)
NE/FP ratio	1.59	1.97	2.32

[a] The reaction mixture contained 40 mM potassium phosphate (pH 8.1), 10 units of AAT, 35 milliunits of CS, and 35 milliunits of MDH whether the enzymes are in their native state or as part of the fusion protein. The transient time is expressed in seconds. [b] Values in parentheses are those obtained in the absence of AAT. The theoretical values are τ = 7.89 s for the native enzymes and τ = 16 s for the fusion protein.

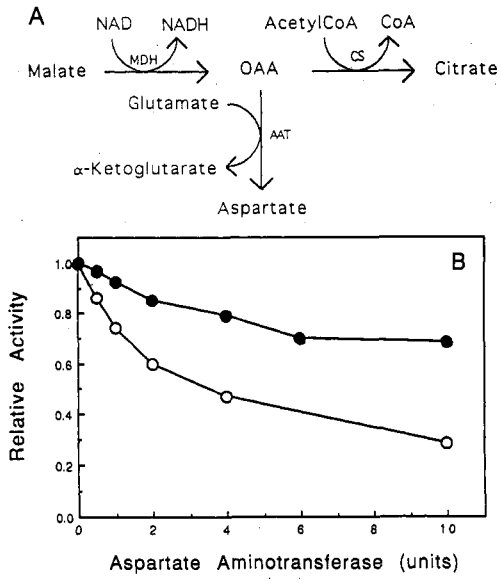

FIGURE 4: (A) Reaction scheme for AAT competition for OAA formed in the coupled MDH/CS reaction. (B) Effect of AAT on the coupled reaction catalyzed by MDH and CS. The values of the initial velocity of the coupled reaction carried out with no AAT are V_0 = 43 μmol of product/min for the fusion protein and V_0 = 40 μmol of product/min for the free enzymes. The corresponding values of k_{cat} are k_{cat} = 9 s^{-1} for the fusion protein and k_{cat} = 11 s^{-1} for the free enzyme. The overall reaction was measured in 40 mM phosphate buffer (pH 8.1). Curves: (●) CS1/MDH1 protein containing 35 milliunits of MDH and 35 milliunits of CS; (○) native MDH1 (35 milliunits) plus native CS1 (35 milliunits).

decreases with increasing AAT concentration but to a lesser extent than for the system based on free native enzymes (Figure 4B).

When the reaction profile for the overall reaction is investigated, it also becomes clear that the two systems behave differently. Reproducible changes in the lag phase were seen at all AAT concentrations. It is possible to calculate the theoretical value of this transient time for each system according to Keleti (1986). Theoretically, the transient time (τ) for the free enzyme system is about 7.99 s while the one for the fusion protein is about 16 s. Experimentally, the τ for the free enzyme system was 7.0 ± 1.5 s and that for the fused system was 4.4 ± 1.0 s (Table 3). These results represent the average of triplicate determinations at seven different AAT concentrations. No difference in lag time was seen either with or without AAT in the case of the native enzymes only or in the case of the fused system only. With lower concentrations of malate, the transient time for the coupled reaction in the presence of 10 units of AAT was markedly shorter with the fused enzyme than with the native free ones (Table 3).

DISCUSSION

Production of Fusion Protein. The fusion protein of CS1/MDH1 can be expressed in *E. coli*. In those strains of *E. coli* lacking either CS or MDH which show an inability to grow on acetate, the transformation with yeast CS1/MDH1 restored growth on acetate. Similar results are reported for yeast mutants in an accompanying paper (Lindbladh *et al.*, 1994).

SDS–PAGE showed the production of a protein in the *E. coli* cells with an M_r of ~90 000–95 000 (expected M_r 88 000) which reacted with both CS1 and MDH1 antibodies.

Kinetics of Fusion Protein. The fusion protein was purified, and SDS–PAGE of the purified sample indicated that in a small amount of the protein proteolysis occurs in the CS1 carboxy terminus just prior to the linker region. On the other hand, the different purification steps and gel filtration experiments indicated no free CS1 and MDH1 in the preparation. Both activities were always coeluted. Thus, though some clipping occurred, the subunits remained bound in the fusion protein. Kinetic characterization of this fusion protein showed a number of changes. Most noticeable was the fact that K_ms for all four substrates for MDH1 (malate, NAD, OAA, and NADH) in the fusion protein were one-half the value of those for the free enzyme. For CS1 all the K_m values in the fusion protein were increasing about 50% compared to the initial value obtained with the free enzyme. This is an important point since the relative values of K_ms for OAA, CS, and AAT are the same for free enzymes and the fusion protein, the "protection" of the OAA intermediate, and cannot be due to a greater affinity of CS for OAA in the fusion protein as compared to the free enzyme. A similar direction of changes in K_ms for the fusion protein β-galactosidase/galactose dehydrogenase has been observed (Carlsson *et al.*, 1994).

The most remarkable observation is that when assayed in the presence of a scavenger enzyme for the OAA intermediate (AAT), the fusion protein showed protection of OAA when compared to the identical system containing free CS1 and MDH1. This is observed even though no change in the OAA K_m for CS1 is observed. Again, these results are consistent with the observations reported for the β-galactosidase/galactose dehydrogenase fusion protein system. In addition, they are in concert with the results of Spivey's group, who showed the sequestration of OAA in a solid-state precipitate of pig CS and pig MDH when probed with AAT (Datta *et al.*, 1985).

We have also observed a decrease in the lag time of the coupled reaction with the fusion protein when compared to that of the reaction with free enzymes. This observation is consistent with those made in other fusion enzyme systems. The fact that the experimental value of the transient time for the fusion protein is 4 times lower than the theoretical value showed how efficient the transfer is from MDH to CS in the fused system compared to the free enzyme system.

Possible Structure of the Fusion Protein. The structures of mammalian pig CS (Remington *et al.*, 1982) and mitochondrial pig MDH (Roderick & Banaszak, 1986) have been determined by X-ray crystallography. Dr. S. J. Remington has determined that the carboxy termini of both subunits of

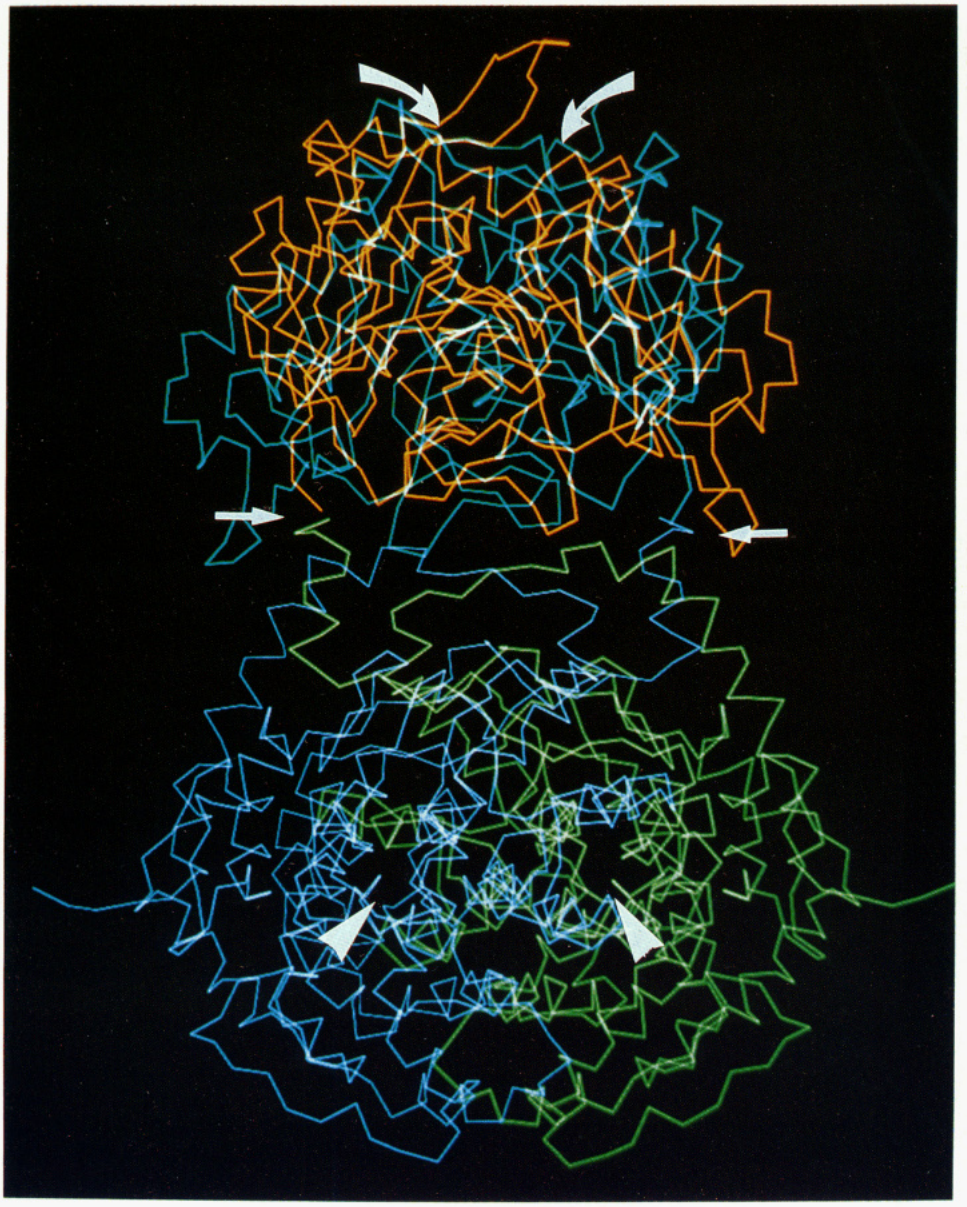

FIGURE 5: Graphic simulation for the pig mitochondrial CS and MDH interaction. Only the backbone of each subunit is shown on this picture. The two monomers of CS are represented in blue and green. The two monomers of MDH are represented in turquoise and orange. The arrows at the bottom indicate CS active sites, those at the top indicate MDH active sites, and those in the middle indicate the interaction resulting from the fusion between the C-terminal end of CS and the N-terminal end of MDH.

pig CS and the amino termini of both subunits of pig MDH can be brought together in a manner such that, with a short linker region, they can be simply docked together as shown in Figure 5. In this configuration the M_r of the fusion protein is ~167 000, and the active sites are at opposite ends of the structure.

Our data are for yeast enzymes for which no X-ray structures exist. However, it is known that considerable sequence homology exists between the yeast and animal enzymes so that it is possible to assume a similar structure for the yeast fusion protein. In fact, three-dimensional structures of homologous enzymes show greater conservation than their primary structure homologies. Extracts of *E. coli* in which the fusion protein is expressed and purified preparations of the fusion protein show overlapping MDH1 and CS1 activities on gel filtration columns with protein fractions having a molecular mass of 167 000 daltons and a small amount with a mass of 300 000–400 000 daltons. It would appear that a dimer of the fusion protein can exist. A simple model for this, based on the known interaction of CS and MDH (Halper & Srere, 1977), is a side by side dimer which would bring the active sites much closer together. We do not know at present the state of the fusion protein *in situ* or even in the assay conditions when the kinetics are examined. We do know, however, that, whatever the structure, MDH-generated OAA is partially sequestered from AAT (less competition) and more available to CS (shortened lag time).

The results reported here confirm previous observations that a kinetic advantage is gained when sequential enzymes are placed in close proximity to each other (Srere *et al.*, 1973) and that if metabolons exist with cells, a benefit would accrue to those cells, in that fluxes could be maintained at lower total substrate concentrations.

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