Electrostatic Channeling of Oxaloacetate in a Fusion Protein of Porcine Citrate Synthase and Porcine Mitochondrial Malate Dehydrogenase†

Konstantin Shatalin,[§,⊥] Sandrine Lebreton,[§,⊥] Magali Rault-Leonardon,[||] Christian Vélot,[⊥] and Paul A. Srere*[,‡,⊥]

Pre-Clinical Science Unit, Dallas VA Medical Center, and Biochemistry Department, University of Texas Southwestern Medical Center at Dallas, 4500 South Lancaster Road, Dallas, Texas 75216

Received September 11, 1998; Revised Manuscript Received November 16, 1998

ABSTRACT: Mitochondrial malate dehydrogenase and citrate synthase are sequential enzymes in the Krebs tricarboxylic acid cycle. We have shown [Lindbladh, C., Rault, M., Hagglund, C., Small, W. C., Mosbach, K., Bülow, L., Evans, C., and Srere, P.A (1994) *Biochemistry 33,* 11692−11698] that a fusion protein of yeast mitochondrial citrate synthase and yeast mitochondrial malate dehydrogenase channels oxaloacetate between the active sites. A Brownian dynamics simulation model of porcine mitochondrial enzymes of citrate synthase and malate dehydrogenase was used [Elcock, A. H., and McCammon, A. M. (1996) *Biochemistry 35,* 12652−12658], showing that a positive electrostatic surface potential between the active sites of the fusion protein could account for the channeling of oxaloacetate we observed with the yeast fusion protein. Since the data were established with a yeast fusion protein and the model was with porcine fusion protein, we have now prepared and studied the porcine fusion protein. The channeling of the oxaloacetate intermediate was the same for the porcine fusion protein as it was for the yeast fusion protein. This channeling behavior is eliminated at high ionic strength. A fusion protein of porcine citrate synthase and porcine cytosolic malate dehydrogenase does not exhibit any channeling of oxaloacetate. A model of the fusion protein with the cytosolic malate dehydrogenase shows no clear positive electrostatic potential surface between the two active sites, thus distinguishing it from the fusion protein with the mitochondrial malate dehydrogenase. These results establish the electrostatic nature of channeling in mitochondrial fusion proteins.

A fusion protein has been prepared previously by linking the carboxyl terminus of yeast mitochondrial citrate synthase (CS1)[1] with a three amino acid linker in-frame to the amino terminus of yeast mitochondrial malate dehydrogenase (Mdh1p)[1] (*1*). The kinetics of the coupled enzyme system indicated that the intermediate oxaloacetate (OAA)[1], produced in the conversion of malate to citrate, was channeled between the active sites of the enzymes in the fusion protein. The crystallographic structures of these two yeast enzymes are not known, although structures of porcine citrate synthase (CS)[1] and porcine mitochondrial malate dehydrogenase (mMDH)[1] are available, and a computer model of a porcine fusion protein could be constructed which was assumed to approximate the structure of the yeast fusion protein. The model showed that the fusion as constructed would give a good fit of the enzymes to produce a dimer of the dimers with the active sites about 60 Å apart. This distance, however, was too great to ascribe the kinetic channeling effects to a proximity of the active sites.

Elcock and McCammon (*2*) showed that Brownian dynamic simulations of the porcine fusion protein model of the CS and mMDH indicate that a positive electrostatic surface exists between the two active sites. These electrostatic forces could account for the channeling of OAA between the two active sites. Their simulations also predicted that the channeling behavior should be sensitive to ionic strength but that some channeling would still exist at 150 mM.

We showed (*3*) that a model of a three enzyme fusion, which includes the carboxyl terminus of porcine mitochondrial aconitase (mACO)[1] to the amino terminus of CS and carboxyl terminus of CS to the amino terminus of mMDH, showed a positive electrostatic surface between the active sites of mACO and CS as well as the one between the fusion CS and mMDH described by Elcock and McCammon (*2*). We also showed that the electrostatic surface potential of cytosolic MDH (cMDH)[1] was quite different than that of mMDH. That difference might account for the difference in behavior of cMDH and mMDH with CS that was observed

† This work was supported by grants from the Department of Veterans Affairs and NSF (MCB-9724922).

* Address for correspondence: Paul A. Srere, Ph.D. (151B), Dallas VA Medical Center, 4500 S. Lancaster Rd., Dallas, TX 75216. Tel: (214) 376-1050. Fax: (214) 372-9534. E-mail: pasrere1@airmail.net.

‡ Dallas VA Medical Center.

§ These authors contributed equally to this paper.

|| Present address: IUT Genie Biologique, Laboratoire Agrobiologie, UPRES EAN° 2152, site Agroparc, BP1207, 84911 Avignon cedex 9, France.

⊥ University of Texas Southwestern Medical Center.

[1] Abbreviations: cMDH, porcine cytosolic malate dehydrogenase; mMDH, porcine mitochondrial malate dehydrogenase; CS, porcine citrate synthase; mACO, porcine mitochondrial aconitase; AAT, aspartate aminotransferase; DTNB, dithionitrobenzoate; OAA, oxalacetate; IPTG, isopropyl β-D-thiogalactoside; SDS, sodium dodecyl sulfate; PEG, poly(ethylene glycol); PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulphonyl fluoride; CS/cMDH, fusion protein of CS and cMDH; CS/mMDH, fusion protein of CS and mMDH; CS1, yeast mitochondrial CS; Mdh1p, yeast mitochondrial MDH; CS1/Mdh1p, fusion protein of CS1 and Mdh1p; SA, specific activity units (mg of protein)$^{-1}$.

10.1021/bi982195h CCC: $18.00
Published on Web 12/31/1998

in earlier experiments where CS and mMDH precipitated in 14% PEG, but CS and cMDH did not (*4*).

The previous experimental work was performed with a yeast fusion enzyme so that *in vivo* characterization of physiological behavior could be performed in corresponding yeast mutants. Even though there is good similarity between the amino acid sequences of yeast CS1 and the porcine CS and between Mdh1p and porcine mMDH, there are enough differences so that it was necessary to study the analogous porcine fusion protein of CS and mMDH. In this study we show that the channeling behavior of the porcine fusion protein, CS/mMDH, is identical to the yeast fusion protein, CS1/Mdh1p. In addition, we show that an increase in ionic strength eliminates the channeling behavior of OAA in the CS/mMDH. Thus, the theoretical predictions of Elcock and McCammon (*2*) are confirmed with the same proteins they used for their Brownian dynamic calculations.

We also show that the CS/cMDH fusion protein cannot channel OAA in the coupled reaction, confirming our earlier prediction (*3*) concerning this fusion protein.

EXPERIMENTAL PROCEDURES

Materials. Ammonium sulfate suspensions of CS (EC 4.1.3.7), mMDH, and cMDH (EC 1.1.1.37) were from Sigma Chemical Co. (St. Louis, MO). NADH, NAD, malate, OAA, DTNB, coenzyme A, and glutamic acid also were from Sigma. Ammonium sulfate suspension of porcine AAT (EC 2.6.1.1) was from Boehringer Mannheim (Indianapolis, IN). rTEV protease was from Gibco BRL (Gaithersburg, MD). PD10 columns and Sephacryl S200 were from Pharmacia (Piscataway, NJ). Ni-NTA-Agarose column was from QIAGEN (Santa Clara, CA). The pig liver tissue was obtained from a local abattoir. Restriction enzyme endonucleases and T4 DNA ligase were supplied by Boehringer Mannheim. Pfu DNA polymerase and a quick change site-directed mutagenesis kit were supplied by Stratagene (San Diego, CA). The RNA extraction kit and first-strand cDNA synthesis kit were from Pharmacia Biotech. Primers for PCR amplification of the CS, mMDH, and cMDH genes were synthesized on a 381-A DNA synthesizer using the phosphoramidite method of Gibco BRL and are listed below.

Primer 1: 5′-GACATCGGCGCCGCTTCTTCCACGAACTTAAAAGAC-3′

Primer 2: 5′-CGGGATCCCTTAGAGTCCACAAGTTTTATCAG-3′

Primer 3: 5′-CCGGATCCGGAGCCAAGGTAGCTGTGCTGGG-3′

Primer 4: 5′-TGAATCGATTCATTTCATGTTCTTGACAAACTCC-3′

Primer 5: 5′-CCGGATCCGGAATGTCTGAACCAATCAGAGTGCTTG-3′

Primer 6: 5′-TGAATCGATTCAGGCAGAGGAAAGAAATTCAAATG-3′

Primer 7: 5′-CACCCCATGTCTCAGCTGAGTGCAGCCATTACAGCCCTCAAC-3′

Primer 8: 5′-GTTGAGGGCTGTAATGGCTGCACTCAGCTGAGACATGGGGTG-3′

Bacterial Strains and Plasmids. Strain *Escherichia coli JM105* (*5*) was used for the propagation and amplification of plasmids. *E. coli* strain BL21/DE3 (*6*) was used as host cell for over-production of fusion proteins. The pNoTA/T7 shuttle vector for the efficient cloning of PCR products was from 5Prime-3Prime, Inc. (Boulder, CO). The bacterial expression plasmid pODC29 (*7*) derived from His-TEV (*8*) was obtained from the laboratory of Dr. Meg Phillips (Department Pharmacology/UT Southwestern Medical Center at Dallas). This vector contains a T7 promoter for the fusion protein and an amino terminal extension of six histidines followed by a highly specific TEV protease site.

Cloning of Genes Encoding Mature Pig CS, mMDH, and cMDH Enzymes. The total RNA was isolated from pig liver tissue. The cDNA was obtained from this total RNA sample by RT-PCR and used as template for PCR amplification of DNA encoding CS, mMDH, and cMDH mature proteins. Primer 1 and primer 2 were used for PCR amplification of the CS gene. Primer 1 contained an *Ehe*I (*Nar*I) site in the 5′ terminal end and corresponded to the sequence of DNA encoding the NH_2 terminus of mature CS protein. Primer 2 contained a *Bam*HI site and corresponded to the 3′ terminal end of the CS gene so that the translational stop codon was removed in the amplified gene. Primer 3 and primer 5 included *Bam*HI sites and corresponded to the sequences encoding amino terminal ends of mature mMDH and cMDH proteins, respectively. Primer 4 and primer 6 contained *Cla*I sites and corresponded to the 3′ terminal ends of the mMDH and cMDH genes, respectively. DNA molecules produced by PCR were cloned into pNoTA/T7 using the prime PCR cloner cloning system from 5Prime-3Prime. The resulting plasmids were termed pNoTA/CS, pNoTA/mMDH, and pNoTA/cMDH.

Construction and Expression of the Different Fusion Proteins in E. coli. The plasmid pNoTA/CS was digested with *Ehe*I and *Hin*dIII. The DNA fragment corresponding to CS was subcloned into the expression plasmid pODC29, previously digested with the same endonucleases. The resulting plasmid was designated pODC29/CS and used for construction of two new plasmids encoding CS/mMDH and CS/cMDH, respectively. For subcloning of mMDH and cMDH genes into plasmid pODC29/CS, the plasmids pNoTA/mMDH and pNoTA/cMDH were digested with *Bam*HI and *Cla*I. DNA fragments corresponding to either the mMDH or cMDH gene were ligated into pODC29/CS previously digested with the same endonucleases. Resulting plasmids were designated as pODC29/CS/mMDH and pODC29/CS/cMDH. They encoded sequences of 16 amino acids (Met-His-His-His-His-His-His-Ala-Glu-Asn-Leu-Tyr-Phe-Gln-Gly-Ala), including the His tag and the TEV protease recognition site, followed by the entire CS gene fused in-frame with a linker-contained sequence, Gly-Ser-Gly, to the NH_2 terminus of either mMDH or cMDH. The obtained plasmids were transformed into *E. coli* strain BL21/DE3 for over-production of fusion proteins.

Nucleotide Sequence Analysis of the Plasmid. DNA sequence analysis of the three constructs, pNoTA/CS, pNoTA/mMDH, and pNoTA/cMDH, was performed using the dideoxynucleotide method (*9*) with double-stranded plasmid DNA (*10*) by Dr. Bill Crider (Department Molecular Transport/UT Southwestern Medical Center at Dallas). One substitution in position 144 was revealed in the CS sequence that resulted in a change of Ala to Val. Site-directed mutagenesis with primers 7 and 8 was used to restore the gene encoding the native CS amino acid sequence described earlier (*11*). The sequences of mMDH and cMDH were

correct and corresponded to the sequences reported earlier (*12*, *13*).

Production and Purification of the Histidine-Tagged CS/MDH Fusion Proteins. The bacterial cultures were grown at 37 °C in Luria-Bertani medium containing ampicillin at 100 μg/mL. At OD_{600} 1, the fusion protein was induced by the addition of IPTG (0.5 mM), and the cells were grown an additional 11 h at room temperature. Cells were harvested by centrifugation at 10000*g* for 10 min. The cell pellets were washed with 50 mM NaCl and centrifuged again at 10000*g* for 10 min. Finally, the cells were stored at −70 °C until the beginning of purification.

For enzyme isolation, 30 g of frozen cells was thawed in 10 mM Tris-HCl buffer (pH 8.5) and 10 mM β-mercaptoethanol (lysis buffer) containing 1 mM PMSF and 1 mM benzamidine (1 g of wet cells/4 mL). The cell suspension was incubated with 10 mg/mL lysozyme for 30 min in ice and sonicated by alternating five cycles of 30 s sonication with 30 s cooling intervals. The lysate was centrifuged for 25 min at 20000*g*, and the supernatant was then applied to a 10 mL nickel-agarose column previously equilibrated with lysis buffer. The column was successively washed with (1) 150 mL of 20 mM Tris-HCl buffer (pH 8.5) containing 100 mM KCl, 20 mM imidazole, 10 mM β-mercaptoethanol, and 10% (v/v) glycerol (buffer A); (2) 30 mL of 20 mM Tris-HCl (pH 8.5) containing 1 M KCl, 10 mM β-mercaptoethanol, and 10% (v/v) glycerol; and (3) 30 mL of buffer A. Bound proteins were eluted with 50 mL of 20 mM Tris-HCl (pH 8.5) containing 100 mM KCl, 500 mM imidazole, 10 mM β-mercaptoethanol, and 10% (v/v) glycerol at a flow rate of 1.4 mL/min. The fractions containing both CS and MDH activities were pooled and precipitated by the addition of 70% solid ammonium sulfate. The pellet was suspended in 0.1 M Tris-HCl (pH 7.5) and applied to a gel filtration Sephacryl S200 column previously equilibrated with 0.1 M Tris-HCl (pH 7.5). A flow rate of 0.5 mL/min was used, and fractions of 0.4 mL were collected. Fractions containing both CS and MDH activities were pooled, concentrated with centriprep 30 (Amicon), and kept in 0.1 M Tris-HCl (pH 7.5) in the presence of 10% glycerol at −20 °C. The Sephacryl S200 column was calibrated using thyroglobulin (670 kDa), bovine γ-globulin (158 kDa), transferrin (75 kDa), phosphorylase B (100 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B12 (1.35 kDa).

Enzyme Assays and Protein Measurements. CS activity was determined using 0.2 mM acetyl-CoA, 0.5 mM OAA, and 1 mM DTNB in 50 mM Tris-HCl (pH 7.7). The reaction was followed spectrophotometrically at 412 nm (*14*) in a total volume of 1 mL. cMDH was assayed either with 0.1 mM NADH and 0.1 mM OAA in 50 mM Tris-HCl (pH 7.7) at 340 nm (*15*) for the reverse reaction or with 4 mM NAD and 10 mM malate in 50 mM Tris-HCl (pH 7.7) for the forward reaction. mMDH activity was determined in 40 mM potassium phosphate (pH 8.1) as described above for cMDH except that the malate concentration was 3 mM. 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 13600 and 6220 M^{-1} cm^{-1} for DTNB and NADH, respectively. One unit of enzyme liberates 1 μmol of product/min. Specific activities (SA) are reported as units per milligram of protein. All reactions were carried out at 25 °C.

Protein concentration was determined with Bio-Rad protein dye assay reagent using bovine serum albumin as a standard.

Removal of Affinity Tags from Fusion Proteins. To determine the effect of the histidine tag, we removed the tag from the purified fusion protein using the TEV protease according to the manufacturer. The hydrolysis was monitored for 3 h at 4 °C. The histidine tag and the protease were then removed from the solution using a PD10 column from Pharmacia. The resulting fusion protein was concentrated by centrifugation using Amicon cells. Glycerol (10%) was added to the solution, and the purified protein was kept at −20 °C.

Coupled Enzyme Reaction. The overall coupled reaction of malate to citrate catalyzed by cMDH and CS for the fusion protein or the free enzymes 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 (pH 8.1). The overall reaction of malate to citrate catalyzed by mMDH and CS, either for the free enzymes or the fusion protein, was assayed (at pH 8.1) as described previously (*1*) except that the malate concentration was 3 mM. A scavenger system for the intermediate OAA was added to the reaction to measure any differences between the system with the fusion proteins CS/MDH and that with equivalent enzyme activities of the free enzymes. Different concentrations of AAT as specified in the text and 4 mM glutamate were used.

Transient Time. The transient time, or the lag phase, is defined as the time required to attain a steady-state condition (*16*). The overall coupled reaction rates from malate to citrate catalyzed by 24 milliunits of mMDH and 52 milliunits of CS either by the fusion proteins or by the free enzymes were assayed as described previously (*1*) except that the malate concentrations were either 2.5 or 0.25 mM and the potassium phosphate concentrations were either 40 or 150 mM. AAT (10 units) and glutamate (4 mM) were added to the reaction mixture when a scavenger system for the intermediate OAA was created. The overall coupled reaction catalyzed by cMDH and CS either as the free enzymes or as the fusion protein was assayed with 20 milliunits of cMDH and 80 milliunits of CS.

Electrophoresis and Immunoblotting. Electrophoreses were carried out with a Bio-Rad minigel apparatus. Electrophoreses under either native or denaturing conditions were performed on 8% polyacrylamide gels. Proteins were stained with Coomassie brilliant blue. The M_r's of the fusion proteins were determined by comparing their relative mobility with those of the standard calibration proteins from Pharmacia-LKB. Proteins separated by PAGE were electrotransferred to nitrocellulose membranes. The filters were subjected to immunoblotting with a rabbit antiserum directed against either CS or MDH. Antibody binding was revealed using anti-rabbit IgG coupled to alkaline phosphatase as described by Sambrook et al. (*17*).

Theoretical Isoelectric Point Determination. The theoretical isoelectric points were determined for both fusion proteins and free enzymes by using the protein characterization tool available on Internet at the address http://hirta.tay.ac.uk/restools/biotools11.html.

Molecular Surfaces and Electrostatic Potentials. Program GRASP (*18*) was used to build molecular surfaces and to

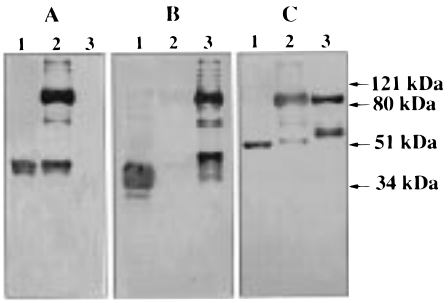

FIGURE 1: Immunoblots of free and fusion enzymes. Free and fusion proteins, after electrophoresis under denaturing conditions and transfer onto nitrocellulose, were probed with antibodies raised against porcine cMDH (A), mMDH (B), or CS (C). Lanes 2 and 3 correspond to 3.5 μg of CS/cMDH and 3.4 μg of CS/mMDH, respectively. Lane 1 corresponds to 0.7 μg of commercial cMDH (A), 0.8 μg of mMDH (B), or 0.5 μg of CS (C), respectively.

Table 1: Purification of CS/MDH Fusion Proteins from *E. coli* Cells

	protein (mg)	CS activity (units)	SA (CS)	MDH activity[a] (units)	SA (MDH)
CS/mMDH					
sonication	3200	4480	1.4	22 400	8
nickel agarose	12.8	541	42	1 794	140
gel filtration sephacryl S2000	2.6	169	65	572	220
CS/cMDH					
sonication	4200	9600	2.3	33 580	8
nickel agarose	20	471	23.5	1510	75.4
gel filtration sephacryl S200	4	280	70	1400	350

[a] The MDH activity was monitored in the reverse reaction from OAA to malate.

calculate electrostatic potentials of both fusion proteins. The crystal structures used are pig CS (*19*), pig mMDH (*20*), and pig cMDH (*21*). Titrable residues were assumed to be in their protonation state at pH 7. The net charge of all Glu and Asp amino acids was set at −1e, while the net charge of Lys and Arg residues was set at +1e. All histidine residues were in their neutral forms. Considering the p*K*s of these residues the net charge is unlikely to be greatly different at pH 8.1. Therefore, the total charges of CS, mMDH, and cMDH are respectively −2e, +6e, and −6e. The ionic strength was set at 0 mM in all of the calculations.

RESULTS

Purification and Characterization of the Fusion Proteins. After induction with IPTG, the cells bearing the recombinant plasmids revealed the presence of either CS/mMDH or CS/cMDH in the crude extract as shown by immunoblotting experiments (data not shown). The antibodies raised against CS recognized both purified fusion proteins, and the antibodies raised against either cMDH or mMDH revealed only the fusion protein containing the corresponding isoform of MDH (Figure 1). No cross reaction was observed. The fusion proteins were purified according to the protocol described in Experimental Procedures. The successive steps of purification are shown in Table 1. The fusion proteins obtained under these conditions were homogeneous on polyacrylamide gel under native conditions (Figure 2A). Few additional bands were detected under denaturing conditions. They may correspond to some proteolytic nicking during the preparation of the fusion protein.

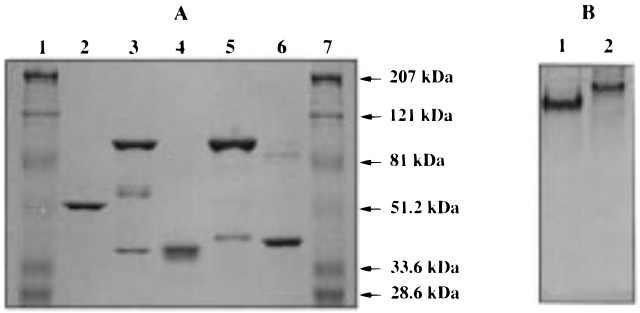

FIGURE 2: Electrophoresis of the fusion proteins. (A) SDS−PAGE of the fusion proteins and the free enzymes. Lanes 1 and 7 correspond to standard proteins of known molecular masses, myosin (207 kDa), β-galactosidase (121 kDa), bovine serum albumin (81 kDa), ovalbumin (51.2 kDa), carbonic anhydrase (33.6 kDa), and soybean trypsin inhibitor (28.6 kDa). Lanes 2, 4, and 6 correspond to 1.5 Fg of free pig CS, 2.5 μg of free pig mMDH, and 2 μg of free pig cMDH, respectively. On lanes 3 and 5, 3.4 μg of CS/mMDH and 3.5 μg of CS/cMDH were applied, respectively. The proteins were stained with Coomassie blue. (B) Native PAGE of the fusion proteins: 3.5 μg of CS/cMDH and 3.4 μg of CS/mMDH obtained from the gel filtration step were applied on lane 1 and lane 2, respectively. The proteins were stained with Coomassie blue.

No difference was observed in the electrophoretic mobility of the two fusion proteins using SDS−PAGE (Figure 2). According to the migration pattern and the calibration curve obtained with standard proteins, the monomers of both purified fusion proteins had a molecular mass of about 84 kDa. These molecular masses corresponded to the association of one subunit of CS (50 kDa) plus one subunit of MDH (mMDH, 34 kDa; cMDH, 36 kDa).

The same chromatographic pattern on gel filtration was obtained with each fusion protein (data not shown). Overlapping CS and MDH activities were found in only one peak with no lower M_r active proteins. After calibration of the column, the molecular mass of this entity appeared to be approximately 170 kDa. As the molecular mass of the monomer was about 85 kDa, the fusion proteins, both CS/mMDH and CS/cMDH, must be dimeric. In some preparations, a protein with a molecular mass of 340 kDa was observed on the elution profile of the Sephacryl S200 column.

The electrophoretic patterns of CS/mMDH and CS/cMDH were slightly different under native conditions (Figure 2B). As the molecular masses of these proteins were the same, as determined on a gel filtration column, this difference in migration represents the different net charge of these two fusion proteins. The theoretical isoelectric points were determined and a value of 7.9 was obtained for CS/mMDH fusion protein while a lower value of 6.6 was calculated for CS/cMDH fusion protein. These theoretical values were in agreement with the migration pattern of both fusion proteins under native conditions. This difference was due to cMDH being much more acidic (pI 6) than mMDH (pI 8.55). The isoelectric point of pig CS is about 6.98.

Kinetics of CS, cMDH, and mMDH in the Fusion Proteins. Both the free enzymes and the enzymes in the fusion proteins followed Michaelis−Menten kinetics. The experiments with the two fusion proteins were performed either with or without the histidine tag, and we found that it did not change the

Table 2: Kinetic Parameters of CS, mMDH, and cMDH as Free Enzymes or Parts of Fusion Proteins

	K_m (AcCoA)[a] (μM)	K_m (OAA)[a] (μM)	SA[a] (units/mg)
CS	7.95 ± 0.68	5.21 ± 0.51	98.75 ± 2.24
CS in CS/cMDH	7.19 ± 0.73	5.38 ± 0.53	68.65 ± 1.87 (114.66 ± 3.13)
CS in CS/mMDH	8.56 ± 0.90	6.00 ± 1.10	76.00 ± 1.7 (126 ± 2.8)[b]

	K_m (malate) (mM)	K_m (NAD) (mM)	SA (units/mg)
cMDH	0.55 ± 0.03	0.31 ± 0.02	36.00 ± 0.8
cMDH in CS/cMDH	0.56 ± 0.13	0.26 ± 0.04	17.40 ± 0.68 (43.49 ± 1.7)
mMDH	0.42 ± 0.04	0.22 ± 0.03	68.50 ± 8.00
mMDH in CS/mMDH	0.29 ± 0.02	0.29 ± 0.01	34.25 ± 2.90 (85± 7)

	K_m (OAA) (μM)	K_m (NADH) (μM)	SA (units/mg)
cMDH	40.3 ± 2.4	23.5 ± 2.1	328 ± 34
cMDH in CS/cMDH	40.5 ± 2.1	37.7 ± 0.4	221.5 ± 20 (554 ± 50)
mMDH	36 ± 4	29 ± 4	907 ± 80
mMDH in CS/mMDH	36.4 ± 0.7	32.5 ± 2	359 ± 26 (897 ± 65)

[a] The activities were followed as described in Experimental Procedures. The K_m and specific activities were determined by fitting all of the curves to the Michaelis−Menten equation. The concentration of free or fusion enzymes was 3 nM in the assay cuvette. The MDH forward reaction was always followed with a concentration 2× higher. The specific activities were calculated per milligram of fusion protein. [b] The specific activities of CS or MDH per milligram of the corresponding enzyme in the fusion protein are indicated in parentheses.

kinetic behavior of the fusion enzymes (data not shown). However, we used only the preparations with the His tag.

The K_m's for acetyl-CoA and OAA in the CS forward reaction in the fusion proteins were measured, and no significant changes were observed either for acetyl-CoA or OAA when compared to the free CS (Table 2). The specific activity of the CS in the fusion protein compared to that of the free enzyme was apparently decreased by about 30% if the value was calculated for the total amount of fusion protein. However, CS protein only represented 60% of the fusion protein so that the specific activity of CS based on CS protein was slightly enhanced in the fusion protein over that of the free enzyme.

The kinetic parameters of mMDH were measured both in the reverse reaction from OAA to malate and in the forward reaction from malate to OAA. The Michaelis−Menten constants for NADH and OAA of mMDH were almost the same in the fusion protein compared to the free enzyme. The K_m of malate decreased about 30%, and the K_m of NAD increased by 25% in the fusion protein (Table 2). Similar to CS activity, a slight increase of the specific activity (per milligram of MDH in the fusion protein) was observed in the fusion protein. We found also that substrate inhibition of mMDH in the reverse reaction by OAA was much stronger for free mMDH than for mMDH in the fusion protein (data not shown).

In the case of the CS/cMDH, a slight decrease in the K_m value for NAD (20%) and an increase (34%) in the K_m of NADH was observed when compared to the free enzyme (Table 2). As was seen previously with CS/mMDH, a higher specific activity was observed for cMDH in the fusion protein when compared to the free enzyme. With cMDH, the substrate inhibition by OAA was much stronger in CS/cMDH than for the free cMDH (data not shown).

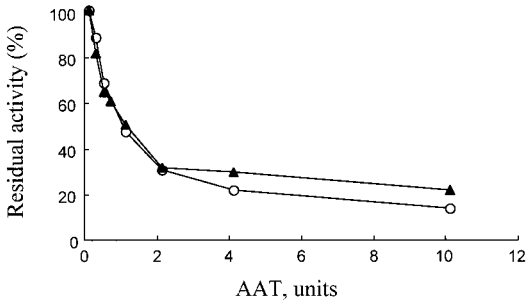

FIGURE 3: Effect of AAT on the coupled reaction catalyzed by CS and cMDH. The overall reaction from malate to citrate was followed in 40 mM potassium phosphate (pH 8.1). CS (45 milliunits) (6 nM) and cMDH (15 milliunits) (8 nM) (forward reaction) were with either with the free enzymes (○) or fused enzymes (▲) (6.9 nM). The initial velocity without AAT was about 12 nmol of product/min for each system.

Coupled Reaction. The overall coupled reaction catalyzed by the fusion proteins and/or by the free enzymes CS and MDH was monitored from malate to citrate as described in Experimental Procedures. To compare the overall reaction catalyzed by the fusion proteins and the free enzymes, we used the same number of units of free or fused enzymes. Different amounts of the OAA trapping enzyme, AAT, were added to the reaction mixtures. This last enzyme is able to compete with CS for the OAA intermediate produced by MDH that forms with glutamate, α-ketoglutarate, and aspartate.

For experiments with CS and cMDH, for both the fusion protein and the free enzymes, a ratio of 3 units of CS for 1 unit of cMDH was used. This ratio corresponds to the activity of purified fusion protein in the conditions described for the measurement of the coupled reaction. For the coupled reaction catalyzed either by CS/cMDH or by the free enzymes, an increase in the concentration of AAT in the reaction mixture caused an inhibition of the coupled reaction (Figure 3). The pattern of inhibition for both was the same. In the presence of 10 units of AAT, only 15−20% of the MDH and CS coupled activity was observed. The fact that the activity of the coupled reaction decreased to the same extent with increasing AAT concentration means that the fusion of the enzymes did not modify the kinetic behavior of the reaction and that no OAA channeling occurred.

The coupled reaction catalyzed by CS/mMDH or free CS and free mMDH was carried out in the presence of a ratio of 2 units of CS for 1 unit of mMDH which corresponded to the ratio of activities in the CS/mMDH. The behavior of the coupled reaction of the CS/mMDH in the presence of increasing trapping enzyme AAT was different than that of free CS and mMDH. The rate of the coupled reaction catalyzed by the fusion protein CS/mMDH was much less inhibited by AAT than the rate catalyzed by the free enzymes (Figure 4). When AAT (10 units) was included in the reaction mixture, only a 50% inhibition of the coupled enzyme activity of CS/mMDH was observed, while an 85% inhibition was observed with free enzymes at this AAT concentration.

Effect of Ionic Strength. The effect of increasing ionic strength on the overall reaction was investigated in the

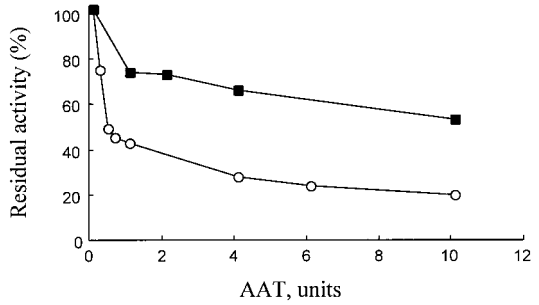

FIGURE 4: Effect of AAT on the coupled reaction catalyzed by CS and mMDH. The overall reaction from malate to citrate was followed in 40 mM potassium phosphate (pH 8.1). CS (30 milliunits) (3.6 nM) and mMDH (15 milliunits) (4.5 nM) (forward reaction) were used with either the free enzymes (○) or fused enzymes (■) (4.5 nM). The initial velocity without AAT was about 10 nmol of product/min for each system.

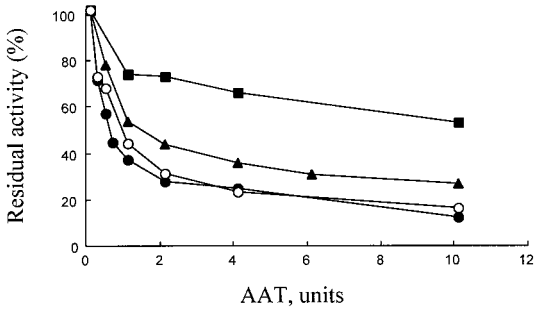

FIGURE 5: Effect of increasing ionic strength on the coupled reaction catalyzed by the fusion protein, CS/mMDH, in the presence of AAT. The overall reaction from malate to citrate was followed in 40 mM (■), 100 mM (▲), and 200 mM (●) potassium phosphate (pH 8.1). CS (30 milliunits) (3.6 nM) and mMDH (15 milliunits) (4.5 nM) (forward reaction) were used. The coupled reaction catalyzed by the free enzymes (○) was run in 40 mM KPO_4 (pH 8.1).

presence of increasing concentrations of AAT. The production of citrate from malate was monitored in buffer containing 40, 100, and 200 mM potassium phosphate for both constructions and for a mixture of the free enzymes (Figure 5). The behavior of fusion proteins was compared to that of free enzymes.

The effect of AAT observed on the coupled reaction monitored with either CS/cMDH or a mixture of the free enzymes was not altered by an increase in the ionic strength of the buffer. The same 80−85% inhibition was observed in the presence of 10 units of AAT (data not shown). With the CS/mMDH fusion protein, a decrease of the protection of OAA was observed with increasing ionic strength (Figure 5). At 200 mM potassium phosphate, the fusion protein CS/mMDH behaves exactly as the free enzymes or the fusion protein CS/cMDH, suggesting that no channeling of OAA produced by MDH occurred at that ionic strength.

Transient Time Measurements. To investigate the proximity effects and the possibilities of substrate channeling, we measured the transient times for coupled reaction catalyzed by either fusion proteins or native enzymes systems, as described under Experimental Procedures. Transient times for a two-enzyme coupled system are, in its simplest form, equal to K_m/V_{max} of the second enzyme. Thus, the expected transient time for the free CS and mMDH (Table 2) coupled reaction is 6.0 s. This can be compared to 5 s measured for the free enzymes. At the lower enzyme concentration, τ (transient time) is calculated as 20.8 s, and the experimental τ was 20.0 s. This agreement may be fortuitous considering other factors of the coupled system which do not meet the criterion upon which the formulation is based. The results of the effect of enzyme concentrations, addition of AAT, and ionic strength on transient times for the CS/mMDH fusion protein and for free CS and mMDH are given in Table 3. One sees first that the transient times for the fusion protein were less at both enzyme concentrations for the fusion protein than for the free enzymes. It should also be noted that a decrease in enzyme concentration caused an increase in transient times since τ is inversely related to V_{max} of CS (*27*). Second, the addition of AAT did not affect the transient times of the fusion protein but did increase it for the free enzyme systems. Third, when the ionic strength is increased from 40 mM P_i to 150 mM KP_i, the transient times for the fusion protein were increased to that of the free enzymes with no concomitant effect on the free enzyme systems. When these experiments are repeated at 0.25 mM malate instead of 2.5 mM malate, no large changes in any of the transient times were observed (data not shown). If the transient times for the CS/cMDH fusion protein are compared to those of free CS and cMDH, no difference was seen at either high or low enzyme concentrations (Table 4).

Table 3: Effect of AAT, High Ionic Strength, and Enzyme Concentrations on the Transient Times of the Overall Reaction for the Fusion Protein, CS/mMDH, or Mixture of the Free Enzymes[a]

enzymes	milliunits of CS/milliunits of MDH	40 mM KP_i	40 mM KP_i + AAT τ (s)	150 mM KP_i
fusion protein	52/24	4.0 ± 0.2	4.4 ± 0.2	5.2 ± 0.2
	15/6.8	14.0 ± 2.0	15.5 ± 1.5	25.5 ± 1.5
free enzymes	52/24	5.0 ± 0.2	6.0 ± 0.2	5.1 ± 0.2
	15/6.8	20.0 ± 1.0	29.0 ± 1.5	23.0 ± 2.0
ratio of free enzymes to fusion proteins	52/24	1.25	1.36	0.98
	15/6.8	1.43	1.87	0.90

[a] The reaction mixture contained 40 or 150 mM potassium phosphate (pH 8.1), 2.5 mM malate, 4 mM NAD, 0.1 mM acetyl-CoA, 0.4 mM DTNB, and either 52 milliunits of CS and 24 milliunits of MDH or 15 milliunits of CS and 6.8 milliunits of MDH whether the enzymes were in their free state or as part of the fusion protein. When AAT (10 units) (100 nM) was used as scavenger enzyme for the OAA, 4 mM glutamate was added to the reaction mixture. There is 4.1 nM (1.2 nM) fusion PCS/mMDH, 5.2 nM (1.5 nM) free PCS, and 5.1 nM (1.47 nM) free mMDH. (Parenthetical numbers are the low enzyme concentration.)

Modeling Studies. The model of docking orientation between CS and mMDH previously described by Elcock and McCammon (*2*) and Vélot *et al*. (*3*) was used to build the molecular surface and calculate the electrostatic potential of the fusion protein CS/mMDH. Despite low amino acid sequence identity (10%) between mMDH and cMDH, the structural similarity is high and both enzymes show the same global shape (*3*). This is consistent with the results of Ian Burbulis (Department Biology, Virginia Tech., Blacksburg) who determined that CS docks almost as well with either cMDH or mMDH (personal communication). We constructed a model of fusion enzyme CS/cMDH just by docking cMDH with CS an orientation similar to that of its counterpart mMDH in the CS/mMDH fusion enzyme model. From the electrostatic profiles shown in Figure 6, it was clear that the fusion protein CS/mMDH was much more positively charged than CS/cMDH. From the net charges of the free enzymes, given in the experimental procedures, we could determine a

Table 4: Effect of Enzyme Concentrations on the Transient Time (Expressed in Seconds) of the Overall Reaction for the Fusion Protein, CS/cMDH, or Mixture of the Free Enzymes[a]

enzymes	milliunits of CS/milliunits of cMDH	τ (s)
fusion protein	80/20	5.2 ± 0.2
	15/3.8	20.5 ± 1.0
free enzymes	80/20	4.9 ± 0.2
	15/3.8	19.5 ± 1.0
ratio of free enzymes to fusion proteins	80/20	0.94
	15/3.8	0.95

[a] The reaction mixture contained 40 mM potassium phosphate (pH 8.1), 2.5 mM malate, 4 mM NAD, 0.1 mM acetyl-CoA, 0.4 mM DTNB, and either 80 milliunits of CS and 20 milliunits of MDH or 15 milliunits of CS and 3.8 milliunits of MDH whether the enzymes were in their free state or as part of the fusion protein. There is 6.9 nM (1.3 nM) fusion PCS/cMDH, 8.1 nM (1.53 nM) free PCS, and 8 nM (1.5 nM) free cMDH. (Parenthetical numbers are the low enzyme concentration.)

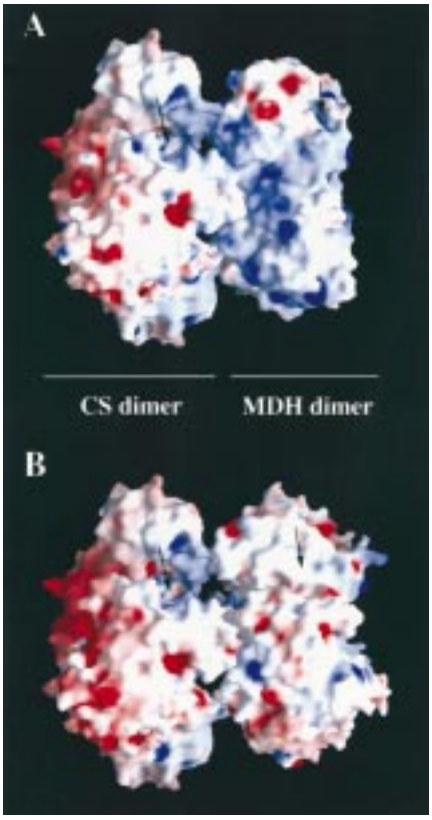

FIGURE 6: Molecular surfaces and electrostatic potentials of CS/mMDH (A) and CS/cMDH (B) complex models. The molecular surfaces and electrostatic potentials are colored according to the electrostatic potential at zero ionic strength. Blue and red areas represent electrostatically positive and negative regions, respectively (limits set at +15 kT). The favored active sites of both enzymes, as described by Elcock and McCammon (*27*), are indicated by a black arrow. This figure was prepared using GRASP (*18*).

net charge of +4e for the dimeric CS/mMDH and −8e for the dimeric CS/cMDH. These values were in agreement with the electrostatic profiles shown in Figure 6.

DISCUSSION

The experimental results presented in this paper showed: (**1**) a porcine CS/mMDH fusion protein could channel OAA on the basis of both a protection of OAA from reaction with an excess of an OAA-requiring enzyme (AAT) and on the basis of a reduction of the transient time of the fusion enzyme coupled reaction; (**2**) both channeling behaviors, the protection of OAA and the decrease in transient time, could be eliminated by increasing the ionic strength of reaction solutions; (**3**) a fusion protein of CS/cMDH did not exhibit either one of the two channeling behaviors seen for CS/mMDH; and (**4**) models of the fusion proteins using the known structures of CS, mMDH, and cMDH showed the already reported positive electrostatic surface potential between the active sites of CS and mMDH, but no similar electrostatic surface existed between CS and cMDH. These results, therefore, further established another mechanism for the channeling phenomenon.

In addition, these results were in agreement with the proposal of McConkey (*22*) on the conserved evolution of the surface potential of proteins. McConkey (*22*) introduced the term "quinary" structure of proteins to indicate transient heterologous polypeptide interactions that occur *in vivo* but cannot be detected *in vitro* after cell disruption. He includes in the list of quinary structures (**1**) ribosomal interactions with initiation, elongation, and termination factors as well as with other proteins; (**2**) nucleosomal histone interaction, high mobility group proteins, and DNA and RNA polymerases; (**3**) cytoskeletal proteins with various proteins such as calmodulin and MAP; and (**4**) protein-modifying proteins with their substrates. He considers that quaternary structure is limited to those complexes which can be isolated intact from cells such as pyruvate dehydrogenase complex, RNA polymerase, and mitochondrial ATPase.

McConkey reached this formulation after comparing by two-dimensional gel electrophoresis the denatured proteins from Hela cells (human) and CHO cells (hamster). He observed that a surprisingly high number of polypeptides of the two cell lines have the same isoelectric points, indicating a conservation of isoelectric points during evolution.

There probably should be two modifications of McConkey's proposal. First, since cellular conditions of volume exclusion and confinement lower the K_d's of protein association (when compared to *in vitro* conditions), it may be incorrect to consider these interactions *in situ* transient. Thus, it will be difficult to assess differences between quaternary and quinary interactions. Second, even though the isoelectric point of a polypeptide may change, it does not necessarily mean that its quinary interaction is lost since a compensatory change in an interacting protein may preserve the interaction.

We previously showed that mitochondrial CSs and mitochondrial MDHs from yeast, porcine, and rat tissues interact similarly in their PEG precipitation behavior and in their ability to bind to the inner surface of mitochondrial inner membrane. Therefore, we felt justified in ignoring species differences in our previous studies and proceeded on the assumption that what was true in one eukaryotic cell would be qualitatively the same in all eukaryotic cells in agreement with the hypothesis of McConkey.

Since CS and mMDH do not form a complex in dilute solution, it has not been possible to observe channeling of the free enzymes, so several strategies have been used to measure kinetics of linked enzymes. One method used was to immobilize the two enzymes (porcine) to Sephadex beads (*23*). This preparation shows a decrease in the transient time of the coupled reaction when compared to the free enzymes.

A second approach was to use lightly sonicated mitochondria which were permeable to substrates but retained bound Krebs TCA cycle enzymes (*24*). In this preparation, the coupled mMDH−CS reaction shows an increased flux when compared to the free enzymes. Spivey's group (*25*) used a PEG precipitate of porcine CS and mMDH and measured the availability of the OAA intermediate to an added enzymatic trap (aspartate amino transferase and glutamate). They found in the precipitate that the OAA is apparently channeled since no inhibition of citrate formation is observed in the presence of a large excess of AAT. We recently confirmed and extended their results (*26*).

Finally, as noted in the Introduction, a fusion protein of the carboxyl terminus of yeast mitochondrial CS1 was linked to the amino terminus of yeast mitochondrial Mdh1p (*1*). This preparation of CS1/Mdh1p shows both a decrease in the transient time of the coupled reaction and evidence of OAA channeling in the presence of AAT. A kinetic model for the reaction by Elcock *et al*. (*27*) yielded theoretical curves that duplicated our experimental results with the yeast fusion protein. We also showed that a model of the fusion protein using the known structures of the porcine CS and mMDH indicated that the active sites are too far apart (60 Å) for the channeling behavior to be explained by the proximity of active sites.

Elcock and McCammon (*2*), using the porcine fusion protein model, showed by Brownian dynamic simulation that a positive electrostatic potential exists between the active sites and could explain the channeling of the negatively charged OAA intermediate. They also predict that an increase in ionic strength of the reaction mixture would decrease the channeling of OAA.

Since differences do exist in sequence, number, and balance of charged amino acid residues between the porcine and yeast CSs and MDHs, we felt it prudent to repeat the yeast fusion protein experiment using a fusion protein that contained the porcine enzymes.

The preparation of the porcine fusion proteins described here was similar to that of the yeast fusion protein except that the use of a His tag on the porcine fusion proteins simplified its isolation. The proteins appeared homogeneous on native gel electrophoresis and on column chromatography. Small amounts of several smaller peptides (∼50 kDa) were seen on SDS−PAGE. These results indicate proteolytic nicking of the fusion protein but that the structure remains intact in the absence of SDS.

The kinetic constants of MDH and CS in the fusion proteins were similar to those of the free enzymes. When the coupled reaction catalyzed by the CS/mMDH fusion protein was measured in the presence of increasing quantities of AAT, the ability of the AAT to trap the OAA intermediate formed with CS/mMDH was less than that seen with a control using free CS and mMDH. These results were almost identical to those seen with the yeast fusion protein CS1/Mdh1p. As before, we interpreted this result to indicate that OAA was channeled between the active site of mMDH and CS.

Another prediction of Elcock and McCammon (*2*) based on their model was that the channeling phenomenon should be sensitive to ionic strength. We have shown here that an increase in ionic strength eliminated the protection of OAA by the fusion protein. This observation is also true for the yeast fusion protein. Thus, the electrostatic model of Elcock and McCammon (*2*) derived from the structure of the porcine CS and mMDH is confirmed experimentally. We can conclude also that the electrostatic model for channeling is true for both the porcine and the yeast fusion proteins.

On the basis of the lack of interactions of CS and cMDH in PEG (*4*) and the difference between the electrostatic surfaces of mMDH and cMDH (*3*), we predicted that a fusion protein of CS and cMDH would not channel OAA. The results presented in this paper are in agreement with that prediction. Moreover, a model of the porcine fusion protein CS/cMDH showed that its electrostatic surface potential was less positive than that of CS/mMDH. This difference confirmed our earlier prediction and provided a physical basis for the difference in the channeling behavior between CS/mMDH and CS/cMDH. Oxaloacetate being negatively charged, its transfer from the MDH active site to the CS active site was more favorable in the case of CS/mMDH. It was also interesting that the active sites of CS and cMDH in the CS/cMDH fusion protein were closer to each other than the active sites in CS/mMDH. This illustrated that proximity did not play a role in this channeling behavior.

While the results presented here did not bear directly on a putative interaction of CS and mMDH *in situ*, they showed that a theoretical basis exists and is experimentally confirmed for the process of metabolite channeling. The concept of channeling has been the subject of dispute for many years, and many accept it only for a few special cases, such as covalent mechanisms (pyruvate dehydrogenase complex) and physical tunneling (tryptophan synthase). Electrostatic surface channeling as illustrated in this work and the work on thymidilate synthetase (*28*) can now be added to that short list of mechanisms. In addition, our results with yeast and porcine enzymes supported the idea of McConkey (*22*) concerning evolutionary conservation of surface charge.

ACKNOWLEDGMENT

The authors wish to thank Virginia Poffenberger for technical assistance and Penny Kerby for manuscript preparation. We also thank Dr. Sarah A. McIntire for her critical review of this paper.

REFERENCES

1. Lindbladh, C., Rault, M., Hagglund, C., Small, W. C., Mosbach, K., Bülow, L., Evans, C., and Srere, P. A. (1994) *Biochemistry 33*, 11692−11698.
2. Elcock, A. H., and McCammon, A. M. (1996) *Biochemistry 35*, 12652−12658.
3. Vélot, C., Mixon, M. B., Teige, M., and Srere, P. A. (1997) *Biochemistry 36*, 14271−14276.
4. Halper, L. A., and Srere, P. A. (1977) *Arch. Biochem. Biophys. 184*, 529−534.
5. Messing, J. (1983) *Methods Enzymol. 101*, 20−78.
6. Studier, F. W., and Moffatt, B. A. (1986) *J. Mol. Biol. 189*, 113−119
7. Osterman, A., Grishin, N. V., Kinch, L. N., and Phillips, M. A. (1994) *Biochemistry 33*, 13662−13667.
8. Parks, P. D., Lenther, K. K., Howard, E. D., Johnston, S. A., and Dougherty, W. G. (1994) *Arch. Biochem. 216*, 413−417.
9. Sanger, F., Nicklen, S., and Coulgon, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463−5467.
10. Wang, Y. (1988) *BioTechniques 6*, 843−845.
11. Evans, C. T., Owens, D. D., Sumegi, B., Kispal, G., and Srere, P. A. (1988) *Biochemistry 27*, 4680−4686.

12. Trejo, F., Costa, M., Gelpi, J. L., Busquets, M., Clarke, A. R., Holbrook, J. J., and Cortes, A. (1996) *Gene 172*, 303−308.
13. Joh, T., Takeshima, H., Tsuzuki, T., Shimada, K., Tanase, S., and Morino, Y. (1987) B*iochemistry 26*, 2515−2520.
14. Srere, P. A., Brazil, H., and Gonen, L. (1963) *Acta Chem. Scand. 17*, S129−S134.
15. Englard, S., and Siegel, L. (1969) *Methods Enzymol. 13*, 99−106.
16. Friedrich, P. (1984) *SUPRAMOLECULAR ENZYME ORGANIZATION*, Pergamon Press, Oxford, U.K.
17. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) in *Molecular Cloning- A Laboratory Manual*, Cold Spring Harbor Laboratory Press, New York.
18. Nicholls, A., Sharp, K. A., and Honig, B. (1991) *Proteins 11*, 281−296.
19. Remington, S., Wiegand, G., and Huber, R. (1982) *J. Biol. Chem. 158*, 111−152.
20. Gleason, W. B., Fu, Z., Birktoft, J. J., and Banaszak, L. J. (1994) *Biochemistry 33*, 2078−2088.
21. Birktoft, J. J., Bradshaw, R. A., and Banaszak, L. J. (1987) *Biochemistry 26*, 2722−2734.
22. McConkey, E. H. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 3236−3240.
23. Srere, P. A., Mattiasson, B., and Mosbach, K. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 2534−2538.
24. Robinson, J. B., Jr., Inman, L., Sumegi, B., and Srere, P. A. (1987) *J. Biol. Chem. 262*, 1786−1790.
25. Datta, A., Merz, J. M., and Spivey, H. O. (1985) *J. Biol. Chem. 260*, 15008−15012.
26. Morgunov, I., and Srere, P. A. (1998) *J. Biol. Chem. 273*, 29540−29544.
27. Elcock, A. H., Huber, G. A., and McCammon, J. A. (1997) *Biochemistry 36*, 16049−16058.
28. Trujillo, M., Donald, R. G. K., Roos, D. S., Greene, P. J., and Santi, D. V. (1996) *Biochemistry 35*, 6366−6374.

BI982195H