Metabolic Studies on *Saccharomyces cerevisiae* Containing Fused Citrate Synthase/Malate Dehydrogenase†

Christer Lindbladh,‡,§ Richard D. Brodeur,§ G. Lilius,‡ Leif Bülow,‡ Klaus Mosbach,‡ and Paul A. Srere*,§

Pure and Applied Biochemistry, University of Lund Chemical Center, Lund, Sweden, and Pre-Clinical Science Unit of the Department of Veterans Affairs Medical Center and Department of Biochemistry, The University of Texas Southwestern Medical Center at Dallas, 4500 South Lancaster Road, Dallas, Texas 75216

Received May 9, 1994; Revised Manuscript Received July 18, 1994®

ABSTRACT: We have constructed two different fusion proteins consisting of the C-terminal end of CS1 fused in-frame to the N-terminal end of MDH1 and HSA, respectively. The fusion proteins were expressed in mutants of *Saccharomyces cerevisiae* in which CS1 and MDH1 had been deleted and the phenotypes of the transformants characterized. The results show that the fusion proteins are transported into the mitochondria and that they restore the ability for the yeast mutants CS1$^-$, MDH1$^-$, and CS1$^-$/MDH1$^-$ to grow on acetate. Determination of CS1 activity in isolated mitochondria showed a 10-fold increase for the strain that expressed native CS1, relative to the parental. In the transformant with CS1/MDH1 fusion protein, parental levels of CS1 were observed, while one-fifth this amount was observed for the strain expressing the CS1/HSA conjugate. Oxygen consumption studies on isolated mitochondria did not show any significant differences between parental-type yeast and the strains expressing the different fusion proteins or native CS1. [3-^{13}C]Propionate was used to study the Krebs TCA cycle metabolism of yeast cells containing CS1/MDH1 fusion constructs. The ^{13}C NMR study was performed in respiratory-competent parental yeast cells and using the genetically engineered yeast cells consisting of CS1$^-$ mutants expressing native CS1 and the fusion proteins CS1/MDH1 and CS1/HSA, respectively. [3-^{13}C]Propionate is believed to be metabolized to [2-^{13}C]succinyl-CoA before it enters the TCA cycle in the mitochondria. This metabolite is then oxidized through two symmetrical intermediates, succinate and fumarate, followed by conversion to malate, oxalacetate, and other metabolites such as alanine. If the symmetrical intermediates randomly diffuse between the enzymes in the mitochondria, the ^{13}C label should be equally distributed on the C2 and C3 positions of malate and alanine. However, if succinate and fumarate are directly transferred with conserved orientation between the active sites of the enzymes succinate thiokinase, succinate dehydrogenase, and fumarase, the labeling of the C2 and C3 positions of malate, oxalacetate, and alanine will be asymmetrical. During oxidation of [3-^{13}C]propionate in parental cells, we observed an asymmetric labeling of the C2 and C3 positions of alanine where the ^{13}C enrichment was significantly higher in the C3 position (C3/C2 = 14.3). Inhibition of succinate dehydrogenase with increasing amounts of malonate resulted in a concentration-dependent decrease in the asymmetric labeling of alanine. When [3-^{13}C]propionate oxidation was performed in the CS1$^-$ yeast cells containing CS1, CS1/MDH1, and CS1/HSA, the CS/HSA transformant displayed significantly decreased asymmetry in the labeling of the C2 and C3 positions of alanine (C3/C2 = 2.9). No significant difference was found between parental cells and the CS1 and CS1/MDH1 transformants. Growth experiments on rich medium did not show any differences between the transformants. On minimal medium, however, the CS1/HSA transformant displayed an increased doubling time. These data show that, in yeast cells containing the CS1/MDH1 fusion protein, symmetrical intermediates are transferred directly from TCA cycle enzyme to TCA cycle enzyme under *in vivo* conditions just as is observed in the parental cell. The data also show that it is possible to alter this effect in the TCA cycle pathway by introduction of a genetically engineered CS1/HSA fusion protein. We also discuss these data in the context of the metabolon hypothesis for the Krebs TCA cycle.

The enzymes which are responsible for catalyzing sequential reactions in several metabolic pathways have been shown to be highly organized. The interactions between the enzymes in these complexes can range from weak and transient interactions to covalently linked systems as found in multifunctional enzymes (Srere, 1987; Srere & Mosbach, 1974). For the enzymes in the Krebs TCA cycle, interactions have been demonstrated between six of eight possible enzymes that are sequential, and all of the enzymes have been found to bind to the inner surface of the mitochondrial inner membrane (D'Souza & Srere, 1983; Robinson & Srere, 1985; Brent & Srere, 1987). It has also been demonstrated that five of the TCA cycle enzymes of *Escherichia coli* can be isolated as a high molecular weight complex (Barnes & Weitzman, 1986). A challenging question to be asked is, "What is the consequence of this enzyme organization for the cell?" There are several possible reasons for organization, including sequestration of intermediates from competing reactions or saving the solvation

† This work was supported by grants from the Department of Veterans Affairs, NIDDK (DK11313), the National Science Foundation (MCB-9117385), the Swedish Research Council for Engineering Sciences (93-815), and the Swedish National Board for Industrial and Technical Development (92-06523).

* Address correspondence to this author at the Department of Veterans Affairs Medical Center [telephone (214) 376-1050; FAX (214) 372-9534].

‡ University of Lund Chemical Center.

§ Pre-Clinical Science Unit of the Department of Veterans Affairs Medical Center and The University of Texas Southwestern Medical Center at Dallas.

® Abstract published in *Advance ACS Abstracts,* September 1, 1994.

0006-2960/94/0433-11684$04.50/0

capacity of the cell (Spivey & Merz, 1989). A number of reports of genetically prepared bifunctional enzymes have demonstrated that it is possible to obtain kinetic advantages in pairs of sequential operating enzymes (Bülow *et al.*, 1985; Bülow, 1987; Ljungcrantz *et al.*, 1989; Lindbladh *et al.*, 1989). For instance, substrate channeling of oxalacetate has been demonstrated in poly(ethylene)glycol precipitates of CS[1] and MDH (Datta *et al.*, 1985). It has also been demonstrated that gently disrupted mitochondria from cells result in a preparation of the TCA cycle enzymes, termed a metabolon, which exhibits kinetic advantages in several coupled enzyme reactions when compared to a soluble mixture of the same enzymes (Robinson *et al.*, 1987). Moreover, investigation of the intermediate substrates and products of the TCA cycle using ^{13}C NMR technology strongly suggests that some kind of substrate channeling exists in the succinyl-CoA synthase to MDH span of the cycle (Sumegi *et al.*, 1990, 1993). Studies have also shown that mutations in the TCA cycle enzymes can change the metabolism in the cycle considerably, even if the mutant enzyme is not involved in the reactions. For instance, cells which lack CS1 or MDH1 lose the ability to grow on acetate. This is unexpected since there are isozymes in the cytoplasm which could act as a shunt for the missing enzyme. Introduction of inactivated CS1 (<1% parental activity) in these mutants restores the ability to grow on acetate, thus suggesting that the CS1 protein (aside from its activity) is important in the overall organization of this metabolic pathway for optimal activity (Kispal *et al.*, 1989).

The ability to produce specific yeast strains by introduction of genetically engineered TCA cycle enzymes into the mitochondria has opened new opportunities to study further the role of enzyme organization in this pathway. The purpose of this paper is to demonstrate that it is possible to make an in-frame gene fusion between the C-terminus of CS1 and the N-terminal MDH1 and to the N-terminus of HSA, respectively, and that the fusion proteins can be expressed and transported into the mitochondria of CS1$^-$ mutant yeast cells. The CS1/HSA fusion was used to see if such a construct might interfere with the TCA cycle metabolon. We also show that there are no significant differences in respiration in the different transformed strains, even if a difference in CS1 activity is observed.

Previous biochemical studies have shown that in most eucaryotic cells [3-^{13}C]propionate is converted to propionyl-CoA and methylmalonyl-CoA, which is converted to [2-^{13}C]-succinyl-CoA. This metabolite is then converted through succinate and fumarate, two symmetrical intermediates, to malate and oxalacetate before it is further oxidized to other metabolites, such as alanine. If tight channeling occurs, the symmetrical intermediates will be transferred between the enzymes before rotational equilibrium is established, i.e., orientation-conserved transfer (Sumegi *et al.*, 1993). This results in an asymmetrically labeled C2 position of malate, which eventually through oxalacetate and pyruvate is further metabolized to [3-^{13}C]alanine. However, if the symmetrical intermediates reach the active sites by diffusion, which allows free rotational equilibrium to occur, both malate and alanine must be equally labeled in the C2 and C3 positions. Our results support previous investigations (Sumegi *et al.*, 1990) and add further evidence for substrate channeling in the succinyl-CoA synthase–succinate dehydrogenase–fumarase–MDH span of the TCA cycle *in vivo* in respiratory-component yeast cells. We also show that it is possible to significantly alter the ^{13}C-labeling pattern of alanine in the yeast transformant which contains the fusion protein CS/HSA in its mitochondria. These results further support the metabolon hypothesis and the fact that effective transfer of the intermediates in this span of the cycle is dependent on an organized enzyme system.

[1] Abbreviations: CS, citrate synthase; MDH, malate dehydrogenase; CS1, yeast mitochondrial CS; MDH1, yeast mitochondrial MDH; HSA, human serum albumin, PDC, pyruvate dehydrogenase complex.

EXPERIMENTAL PROCEDURES

Strains and Plasmids. *E. coli* JM105 (Messing, 1983) was used as the host cell when the fusion proteins were constructed. Plasmid YEp352/CS1 was obtained originally from Drs. L. Guarente, (MIT, Cambridge MA) and M. Rosenkrantz (Virginia Commonwealth University, Richmond, VA), and plasmids Blue Scribe pMDH1 (Thompson *et al.*, 1988) and pJDB207-HSA encoding MDH and HSA, respectively, have been described before (Kàlmàn *et al.*, 1990). Oligonucleotide synthesis for PCR amplification of the genes encoding CS1, MDH1, and HSA was performed on a 381A DNA synthesizer (Applied Biosystems, Foster City, CA), using the phosphoramidite method (Beaucage & Caruthers, 1981) at the Molecular Biology Core Facility (University of Lund, Lund, Sweden). The oligonucleotides were purified on a MonoQ column prior to use. The sequences are

primer 1	5′-CCATGGGAGCTCCCGATACTACTCGACTTATCCGAC-3′
primer 2	5′-GCAGCTGGATCCGTTCTTACTTTCGATTTTCTTTACCA-3′
primer 3	5′-CAGAAAGGATCCGGATATAAAGTGACTGTTTTGGGTGCA-3′
primer 4	5′-GACATCCTGCAGCTATTTACTAGCAACAAAGTTGAC-3′
primer 5	5′-CATGTAGGATCCGGAGACGCTCACAAGTCTGAAGTCGCT-3′
primer 6	5′-GACCGACTGCAGCTATTACAAACCCAAAGCAGCTTG-3′

All enzymatic reactions were carried out as recommended by the manufacturer (Boehringer Mannheim, Indianapolis, IN), and all recombinant manipulations were done according to Maniatis *et al.* (1982).

The CS1$^-$ strain of yeast used in this study originated from the parental strain PSY142 (Kispal *et al.*, 1989). The MDH1$^-$ genotype (Steffan *et al.*, 1980) and the double mutant CS1$^-$MDH1$^-$ from a SG7 parental strain was supplied by Dr. L. McAlister-Henn (University of Texas Health Science Center, San Antonio, TX). These yeast cells were transformed with the final plasmid constructions (Ito *et al.*, 1983) encoding the fusion proteins and the URA gene. Transformed cells were selected by their ability to grow on minimal medium, which contained no uracil, and their strains were characterized.

Media. Bacterial cells were grown in LB medium containing 100 μg/mL ampicillin. Complete medium (YPGal) for growth of yeast contained 1% yeast extract (Difco, Detroit, MI), 2% Difco peptone, and 2% galactose. Minimal medium contained 0.85% yeast nitrogen base (Difco), 2.5% ammonium sulfate, adenine (33 μg/mL), arginine (33 μg/mL), histidine (33 μg/mL), methionine (33 μg/mL), tryptophan (33 μg/mL), isoleucine (50 μg/mL), lysine (50 μg/mL), tyrosine (50 μg/mL), phenylalanine (83 μg/mL), leucine (100 μg/mL), and valine (250 μg/mL). Acetate (0.5%), along with 50 mM MES (pH 5.5), or glucose (2%) was used as a carbon source to make up the final media solutions YNBA (uracil) and YNBD (uracil), respectively.

Isolation of Mitochondria. The method used was essentially that of Daum *et al.* (1972). Cells were grown overnight in 50 mL of YNBD (uracil) and 40 mM potassium phosphate (pH 7.0). The preculture was added to 500 mL of YPGal and 20 mM potassium phosphate (pH 7.0) and grown for

approximately 20 h. The cells were harvested by centrifugation for 5 min at 2500*g*, 4 °C. The cells (typically 6–8 g) were suspended in 80 mL of 100 mM Tris-HCl (pH 9.4) and 10 mM DTT and incubated for 10 min at 30 °C. After centrifugation the cells were washed once with 50 mL of 1.2 M sorbitol and 20 mM potassium phosphate buffer (pH 6.8), followed by digestion of the cell wall for 2 h in 30 °C in the same buffer containing 3 mg of lyticase/g per cell. After digestion the cells were washed in 1.2 M sorbitol. All subsequent steps were carried out at 0 °C. The cells were suspended in 0.55 M mannitol, 20 mM potassium phosphate buffer (pH 6.8), 0.1% BSA, 1 mM PMSF, 1 mM benzamidine, and 2.4 units of aprotinin, followed by gentle homogenization to break up the cells. Cell debris was removed by centrifugation at 2000*g* for 5 min, followed by a final centrifugation at 17000*g* for 5 min to pellet the mitochondria. Isolated mitochondria were typically suspended in 200–300 μL of 0.55 M mannitol, 20 mM potassium phosphate buffer (pH 6.8), and 0.1% BSA, which was also used as the assay buffer.

Respiration Studies. Oxygen consumption was determined using the polarographic assay. Briefly, a Clark electrode coupled to a biological oxygen monitor (YS1 Scientific, Yellow Springs, OH) was immersed in a 0.6-mL chamber containing 0.6 M mannitol and 1 mg/mL bovine serum albumin and buffered with 20 mM potassium phosphate (pH 7.4). Substrate concentrations were used as described previously (Ohnishi *et al.*, 1966).

^{13}C NMR Studies. Packed washed yeast cells (2 g) were resuspended in 10 mL of minimal medium (yeast nitrogen base; Difco) containing 0.5% ammonium sulfate and 5 mg of [3-^{13}C]propionate (sodium salt) (4.5 mM). The resuspended cells were incubated at 30 °C for 30 min with vigorous shaking to supply oxygen. The reaction was terminated by addition of perchloric acid to washed centrifuged cells to a final concentration of 4%. The supernatant fraction was neutralized with KOH, centrifuged to remove salt, and lyophilized. The resulting powder was resuspended in 0.65 mL of D_2O and microfuged to remove any residual particulates.

High-resolution ^{13}C NMR spectra were recorded on a GN-500 spectrometer at 11.75 T. The number of scans used for each sample varied between 2000 and 8000. All spectra reported in this work were acquired using a 45° carbon pulse and a 6-s delay between pulses to ensure nonsaturating conditions. All samples were maintained at 25 °C during data acquisition. The ^{13}C resonances were quantitated using NMR286 software (Softpulse). All noise levels were normalized and peak heights used to determine the fractional enrichments of the carbon pools.

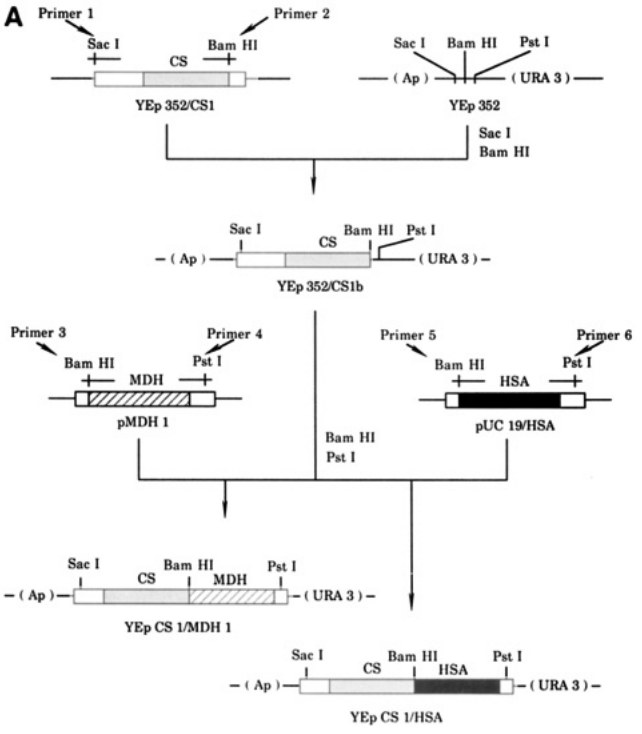
Growth Studies. Growth of yeast cells was followed as described previously (Kispal *et al.*, 1988).

Determination of Enzyme Activities. CS (Srere *et al.*, 1963), MDH (Englard & Siegel, 1969), aconitase (Fansler & Lowenstein, 1969) fumarase (Hill & Bradshaw, 1969), isocitrate dehydrogenase (Plaut, 1969), and PDC activities (Shinman & Bass, 1981) were determined on isolated mitochondria according to standard procedures. Protein concentration was determined by the Bradford method (Bradford, 1976).

Gel Electrophoresis and Immunoblot. PAGE electrophoresis and immunoblotting were carried out as described previously (Kispal *et al.*, 1989).

RESULTS

Construction of Fusion Proteins. To remove the translational stop codon of the CS1 gene and make the fusion proteins,



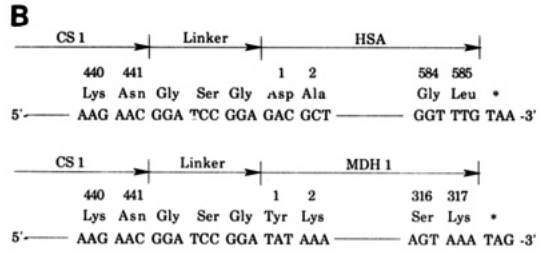


FIGURE 1: (A) Schematic construction of shuttle vectors YEp352 CS1/MDH1 and YEp352 CS1/HSA, encoding the two fusion proteins. Plasmids are not drawn to scale. Full details about the cloning are given in the text. Abbreviations: CS1, citrate synthase; MDH1, malate dehydrogenase; HSA, human serum albumin; AP, β-lactamase. (B) Scheme of linker regions.

the gene including the promoter was cloned into shuttle plasmid YEp352. Two primers for PCR amplification of the CS1 was designed. Primer 1 had a *Sac*I site in the 5′-terminal end and hybridized approximately 240 bases upstream of the first codon encoding the first amino acids in the signal sequence of CS1. Primer 2 contained a *Bam*HI site and hybridized to the 3′-terminal end of the CS1 gene so that the translational stop codon was removed in the amplified gene. The gene was amplified by PCR from the YEp352/CS1, digested with *Sac*I and *Bam*HI, and inserted into plasmid YEp352 previously digested with *Sac*I and *Bam*HI. This resulting plasmid, YEp352/CS1b, was used in the construction of the two fusion proteins, CS1/MDH1 and CS1/HSA (Figure 1A). Two new primers for PCR amplification of MDH1 were synthesized using Blue Scribe pMDH1 as a template. Primers 3 and 4 encoded a *Bam*HI site and a *Pst*I site, respectively, and hybridized to the 5′-terminal and the 3′-terminal ends of the MDH1 gene. The amplified gene was digested with *Bam*HI and *Pst*I and inserted downstream of the CS1 gene in plasmid YEp352/CS1b. The resulting plasmid, YEpCS1/MDH1, encoded the entire CS1, a linker (Gly-Ser-Gly) followed by the entire MDH1 (CS1/MDH1) (Figure 1B). Similar strategy was followed when plasmid YEpCS1/HSA was constructed using primers 5 and 6 and a template. This plasmid encodes the entire CS1, a linker (Gly-Ser-Gly)

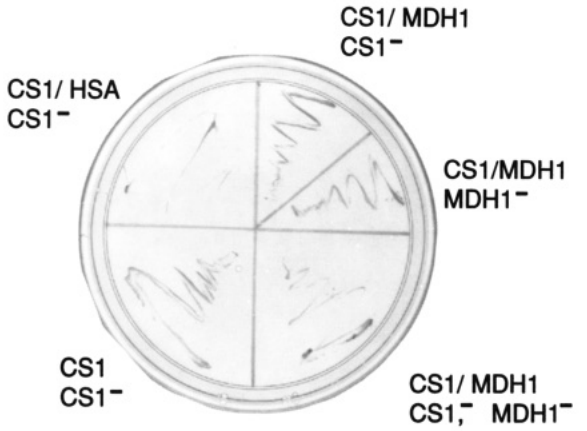


FIGURE 2: Different strains grown on minimal medium with acetate (YNBAc–uracil). The strains are, from the top left, plasmid YEp352 CS1/HSA in PSY142 CS1⁻ genotype, plasmid YEp352 CS1/MDH1 in PSY142 CS1⁻ genotype, plasmid YEp352 CS1/MDH1 in SG7 MDH1⁻ genotype, plasmid YEp352 CS1/MDH1 in SG7 CS1⁻ MDH1⁻ genotype, and plasmid YEp352/CS1 in PSY142 CS1⁻ genotype.

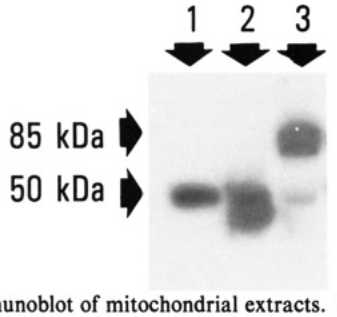


FIGURE 3: Immunoblot of mitochondrial extracts. Lanes: 1, pure CS1; 2, extract of PSY142 mitochondria; 3, extract of PSY142 CS1⁻/CS1/MDH1.

followed by the entire HSA (CS1/HSA).

Growth on Acetate. The yeast mutants CS1⁻ and MDH1⁻ and the double mutant CS1⁻MDH1⁻ have previously been shown not to grow on acetate as the sole carbon source (Kispal *et al.*, 1988). However, when the CS1⁻ yeast cell was transformed with the constructed plasmids encoding CS1/MDH1, CS1/HSA, and CS1, all transformants had the ability to grow on acetate. This was also seen when the MDH1⁻ mutant and the double mutant CS1⁻MDH1⁻ were transformed with the plasmid encoding CS1/MDH1 (Figure 2). These results agree with earlier observations that it is possible to restore the ability of CS1⁻ yeast cells to grow on acetate when CS1 is introduced into this genotype either as an active enzyme or as an inactive enzyme (Kispal *et al.*, 1989).

Further evidence for transport of the CS1/MDH1 fusion protein into the mitochondria comes from Western blotting experiments of isolated mitochondria (Figure 3). The molecular weight for the fusion protein agrees with those of the theoretically calculated value. Similar results were observed with the CS1/HSA fusion protein (not shown).

Mitochondrial CS1 and MDH1 Activities. The activities of CS and MDH and of other Krebs citric acid cycle enzymes were determined in the mitochondria of parental strain and in the transformed CS1⁻ yeast strains producing the fusion proteins (Table 1). The activities of MDH were found to be similar in all genotypes, although slightly higher in CS1⁻ yeast cells expressing the CS1/MDH1 fusion protein. The CS1 activity was, however, found to change considerably. CS1 activity for the parental type agreed with previous data and was found to be similar to the CS1/MDH1-transformed yeast

Table 1: Citrate Synthase and Malate Dehydrogenase Activity in Isolated Mitochondria[a]

strain	CS1 activity (units/mg)	MDH1 activity (units/mg)	CS1 activity/ MDH1 activity
parental, PSY142	1.5	1.0	1.5
YEp352 CS1 in PSY142 CS1⁻	15	1.0	15
YEp352 CS1/ MDH1 in PSY142 CS1⁻	1.4	1.2	1.2
YEp352 CS1/ HSA in PSY142 CS1⁻	0.3	1.0	0.3

[a] Yeast cells were grown on rich medium (YPGal). Mitochondria were isolated as described in Experimental Procedures.

Table 2: Krebs TCA Cycle Enzymes in Transformant Mitochondria

strain	enzyme SA (milliunits/mg of protein)				
	PDC	FUM	ACON	SDH	ICDH
parental PSY142	74 ± 5	386 ± 3	356 ± 20	75 ± 12	217 ± 3
YEp352 CS1/ MDH1 in PSY142 CS1⁻	100 ± 0.8	991 ± 58	484 ± 14	62 ± 10	344 ± 15
YEp352 CS1/ HSA in PSY142 CS1⁻	69 ± 2	755 ± 10	305 ± 2	82 ± 16	144 ± 45

CS1⁻ strain. The strain with CS1 had an increased CS1 activity which resulted in a CS1/MDH1 ratio of 15. This is about 10-fold higher than the ratio found for the parental strain. The CS1/HSA strain, on the other hand, displayed a 5-fold lower CS1 activity and had a CS1/MDH1 ratio of 0.3, which is lower than the other strains. In other experiments other TCA cycle enzymes were also measured in two transformants and found to agree with previous investigations (Kispal *et al.*, 1989) on the parental strain, PSY142 (Table 2).

Respiratory Characteristics of Mitochondria. The oxidation of citrate and α-ketogluarate was measured in coupled mitochondria from the various transformed strains (Table 3). This shows the O_2 uptake with ADP (the ratio of states 4 and 3 in respiring coupled mitochondria). These oxidation rates were compared with NADH oxidation rates to adjust for variations in the mitochondrial preparations. Oxidation of external NADH by the mitochondria is carried out with a dehydrogenase located toward the outer surface of the inner membrane (vonJagow & Klingenberg, 1970). Although some variations are seen from strain to strain, the significant measurements of the respiratory control ratio (RCR) and the P:O ratio are apparently unaffected. The amount of ATP formed per oxygen (P:O) in yeast is ideally 2, but due to various experimental factors this is rarely obtained (Zubay, 1983).

In previous investigations the same respiratory ratio for citrate and α-ketoglutarate was found to be decreased in isolated mitochondria of the CS1⁻ mutant yeast strain (Kispal *et al.*, 1989). Our conclusion is therefore that the fusion proteins and the native CS1 are able to restore the mitochondrial function at these positions of the TCA cycle.

In Vivo ^{13}C NMR. The metabolism of [2-^{13}C]- and [3-^{13}C]-propionate was studied in NMR *in vivo* in parental yeast cells (panels A and B of Figure 4) and with [3-^{13}C]propionate in CS1⁻ mutant yeast cells transformed with CS1 and different genetically prepared fusion proteins, CS1/HSA and CS1/MDH1, respectively (panels A–C of Figure 5). First, control studies in parental cells were performed on yeast grown in

Table 3: Respiratory Characteristics of Mitochondria from Transformed Yeast Strains[a]

strain	oxidation ratio		QO_2 NADH	RCR cit	P:O	
	cit:NADH	αKG: NADH			cit	αKG
parental PSY142	0.40	0.73	538	2.15	0.71	0.90
YEp352 CS1/HSA in PSY142 CS1⁻	0.93	0.55	408	2.71	0.92	0.93
YEp352 CS1/MDH1 in PSY142 CS1⁻	0.81	0.69	333	2.47	0.94	0.80
YEp352 CS1 in PSY142 CS1⁻	0.64	0.66	175	3.05	0.86	0.83

[a] The cells were grown in rich medium (YPGal) and the mitochondria isolated as described in Experimental Procedures.

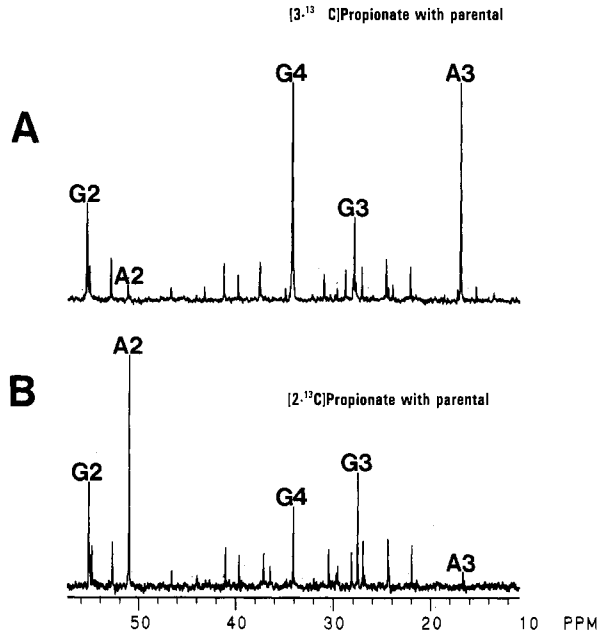


FIGURE 4: ^{13}C NMR spectra of extracts of parental cells grown in [3-^{13}C]propionate (A) and [2-^{13}C]propionate (B). G2, G3 and G4 are the 2, 3, and 4 carbons of glutamate, respectively. A2 and A3 are the 2 and 3 carbons of alanine.

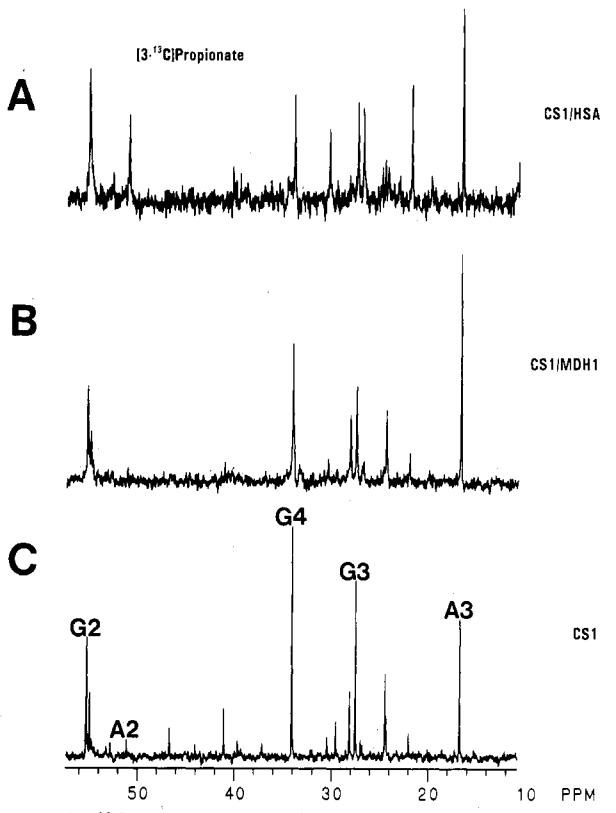


FIGURE 5: ^{13}C NMR spectra of extracts of PSY142 CS1⁻ cells transformed with CS1/HSA (A), CS1/MDH1 (B), or CS1 (C) and grown in [3-^{13}C]propionate. Abbreviations are the same as in Figure 4.

pH-balanced minimal medium containing [3-^{13}C]propionate in the presence of 0, 50, and 500 mM malonate, respectively (Figure 6). The ratio of ^{13}C in the C2 and C3 positions of alanine was determined. When parental yeast was grown with [3-^{13}C]propionate without malonate, the ratio of ^{13}C in the C2 and C3 positions in alanine was 0.070 (Table 4, Figures 4A and 6C). This suggests that the ^{13}C from [3-^{13}C]propionate had either been converted to alanine in a direct pathway or had been transferred through the symmetrical TCA cycle intermediates in the mitochondria, succinate and fumarate, in a highly conserved orientation. If the oxidation is through the Krebs cycle and if there had been free diffusion of these intermediates, the ^{13}C label should be equally distributed in the C2 and C3 positions of malate and oxalacetate and in metabolites derived from these, such as alanine. The ratio of C2 and C3 was used to calculate the percentage of tumbling of the intermediate symmetrical metabolites, succinate and fumarate. If these are allowed to freely rotate, the ratio in these positions will be 1, corresponding to 100% tumbling. Using this definition and assuming a linear relation between the ratio of C2/C3 and the amount of rotational diffusion of the symmetric immediates, only 14% of the intermediates had tumbled, thus leaving 86% of the intermediates to be transferred in a conserved orientation. When oxidation of [3-^{13}C]propionate was carried out in the presence of 50 and 500 mM malonate, the distribution pattern of ^{13}C in alanine was significantly changed. A malonate dose-dependent increase in the tumbling of the symmetrical intermediates was seen (Table 4). Since malonate inhibits succinate dehydrogenase, a larger pool of succinate is formed which can exchange with those succinate molecules associated with the active site of succinate thiokinase and succinate dehydrogenase. All together, this increases the chances of randomization of the ^{13}C label and thus the percentage of tumbling. Moreover, at 500 mM malonate, the [3-^{13}C]propionate is not metabolized completely (Table 4). This suggests that the flux of the TCA cycle is severely reduced, thus allowing almost 100% randomization of the ^{13}C label in the symmetrical intermediates. These studies indicate that there are apparently no important Krebs cycle independent alternative pathways to metabolize propionate in yeast cells since if an energy-dependent non-Krebs cycle pathway were involved, no *change in ratio* would be seen, only a diminution in oxidation rate. These results could all be repeated using [2-^{13}C]propionate (data not shown).

[3-^{13}C]Propionate oxidation was next investigated in CS1⁻ mutant yeast cells which had been transformed with plasmids expressing native CS1 and the fusion proteins CS1/MDH1 and CS1/HSA, respectively. Oxygen consumption studies on isolated mitochondria did not show any significant differences between the different genotypes. When the cells were grown on minimal medium containing [3-^{13}C]propionate, no significant differences could be seen in the labeling pattern of alanine for parental yeast and the CS1 and CS1/MDH1 transformants (Table 5). The ratios all corresponded to a

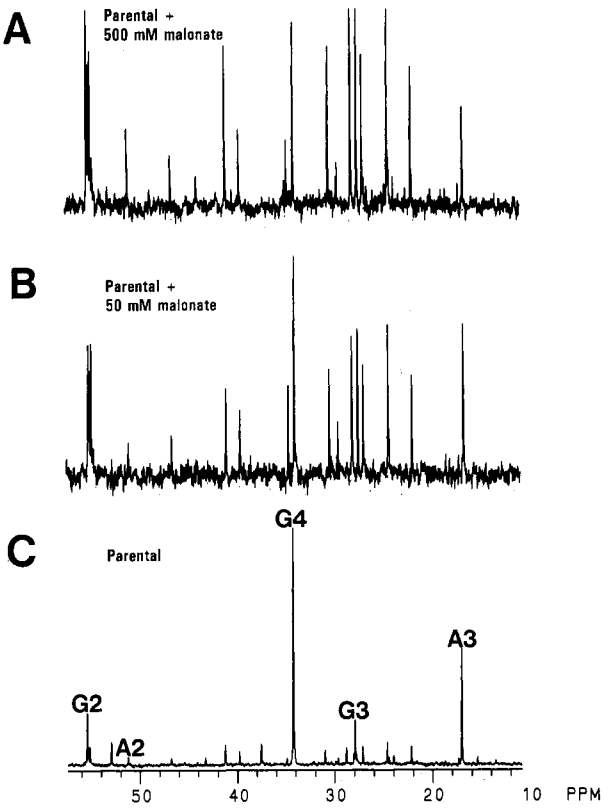


FIGURE 6: ^{13}C NMR spectra of PSY142 cells (parental) grown in 500 (A), 50 (B), or 0 (C) mM malonate. Same conditions as in Figure 5.

Table 4: Change in Ratio of ^{13}C in the C2 and C3 of Alanine after Incubation of Yeast Cells with [3-^{13}C]Propionate[a]

malonate (mM)	C2/C3 of alanine	% tumbling	C3–propionate
0	0.070	14	not present
50	0.20	34	not present
500	0.87	94	present

[a] PSY142 yeast cells were incubated with [3-^{13}C]propionate, and ^{13}C in alanine was determined as described in Experimental Procedures.

Table 5: Ratio of ^{13}C in C2 and C3 of Alanine in Transformed Yeast Cells[a]

transformed plasmid	C2/C3	% tumbling	propionate
parental, PSY142	0.070	14	not present
YEp352 CS1 in PSY142 CS1$^-$	0.086	16	not present
YEp352 CS1/MDH1 in PSY142 CS1$^-$	0.060	12	not present
YEp352 CS1/HSA in PSY142 CS1$^-$	0.344	52	present

[a] PSY142/CS1$^-$ yeast cells were transformed with plasmids containing the indicated genes. After incubation with [3-^{13}C]propionate the amounts of ^{13}C in the C2 and C3 of alanine were determined as described in Experimental Procedures.

tumbling of the symmetrical TCA cycle intermediates of 12–16%. However, in the CS1/HSA genotype, the metabolism of [3-^{13}C]propionate was significantly changed. The C2/C3 ratio of alanine was increased, corresponding to 52% tumbling of the symmetrical intermediates. In contrast to the other transformants, the CS1/HSA transformant was also found to metabolize propionate more slowly since [3-^{13}C]propionate was still present in the cells after the standard incubation period. This further suggests that the presence of the CS1/HSA fusion protein in the mitochondria decreases the flux of the TCA cycle and thus allows the intermediates to diffuse and randomize in analogy with metabolism of [3-^{13}C]-propionate in the presence of 500 mM malonate. The NMR spectra summarized in Tables 4 and 5 are shown in Figures 5 and 6.

Table 6: YNB + 2% Raffinose

cells	lag time (h)	doubling time (h)	extent of growth (30 h)[a]
CS1$^-$	3.0	4.0	×5.6
CS1$^-$/CS1	3.0	3.0	×7.4
CS1$^-$ + CS/MDH	3.0	2.9	×6.8
CS1$^-$ + CS/HSA	4.0	3.5	×5.1

[a] This is the ratio of OD at 30 h compared to 0 time OD.

Growth Studies. The yeast fusion protein transformants were also investigated for their growth properties on different substrates to see whether any important differences could be obtained. The genotypes were grown on rich media and on minimal media containing different carbon sources. Growth on a rich medium (YPD) showed only small differences in doubling time between the transformants despite differences in the specific activities of CS1 and MDH1 in the mitochondria (data not shown). However, when glucose was replaced by raffinose (YNBR), the CS1/HSA transformant displayed a slightly lengthened doubling time (Table 6). This observation was also seen when the transformants were grown on minimal medium containing acetate or glycerol as the sole carbon source.

DISCUSSION

In this paper we describe the construction of two different fusion proteins, CS1/MDH1 and CS1/HSA, which have been constructed by in-frame fusion of respective genes with a Gly-Ser-Gly linker. The gene fusion technique results in well-defined enzyme conjugates and offers new possibilities to further investigate the metabolon hypothesis *in situ* in living cells. The results presented here show that the fusion proteins are expressed and transported into the mitochondria of mutant yeast cells (CS1$^-$, MDH1$^-$, and CS1$^-$MDH1$^-$ mutants) as judged by their ability to grow on acetate, which is indicative for their activity in the mitochondria.

Further characterization of the different transformed strains showed that the activity of CS1 was increased about 10 times in the CS1$^-$ mutant expressing native CS1, relative to parental yeast, and decreased about 5 times relative to parental strain in the CS1/HSA strain. This does not necessarily mean that the flux in the TCA pathway is greatly affected. For instance, the activity of each individual enzyme in the tryptophan synthesis pathway has been up- and down-modulated using genetically engineered yeast to investigate the effect of the overall flux in the pathway (Niederberger *et al.*, 1992). The results demonstrated that yeast cells containing greatly reduced activity (<25% of normal) or dramatically increased activity (10–50-fold) of each enzyme did not change the tryptophan flux. However, when all five enzymes were simultaneously increased, a substantial increase in the tryptophan flux was observed. Similar observations on the lack of change in flux have also been made in the glycolytic pathway of yeast (Schaff *et al.*, 1989) where individual enzymes were increased. In a previous study we have found that the CS1 activity in parental cells is about 30-fold higher than the observed respiratory rate and that the CS1 activity can vary considerably without affecting the oxygen consumption rate (Grigorenko *et al.*, 1990). Our results on oxygen consumption agree with this study in that we did not find any significant differences between the different strains and parental, even if they displayed a

CS1 activity that varied over a wide range (5-fold down to 20-fold up) compared to the parental mitochondria.

Substrate channeling has been reported for several metabolic pathways in which evidence for interacting consecutive enzymes exists (Srere, 1987). Examples include enzymes coimmobilized on solid matrixes (Mosbach & Mattiasson, 1970; Srere *et al.*, 1973), genetically fused enzymes (Bülow, 1987; Ljungcrantz *et al.*, 1989), and systems consisting of permeabilized cells (Clegg & Jackson, 1990). These models have all given valuable information of how consecutive enzymes may work. However, substrate channeling has not been determined with satisfaction *in vivo* in the natural milieu in living cells because of the difficulties in the design and execution of proper experiments. *In vivo* experiments are important since the milieu in the cell usually is very crowded and differs from conditions attainable *in vitro*. Thus, the protein concentration in the mitochondrial matrix has been estimated to be over 50% (w/w) (Hackenbrock, 1968), which is near the value found for very close packing of identical spheres. This high protein concentration may have a number of effects on the enzymes in the matrix such as drastically shifting the equilibrium for enzyme–enzyme association toward complex formation (Minton, 1990), which in turn may affect the transfer mechanism of the substrate.

Our ^{13}C NMR data agree with the results and conclusions of Sumegi *et al.* (1990) and add further evidence supporting this type of transfer phenomenon. We show, as they did, that the oxidation of [3-^{13}C]propionate *in vivo* in respiring yeast cells is metabolized through the Krebs TCA cycle through two symmetrical intermediates (succinate and fumarate) in an orientation-conserved manner to malate which yields asymmetrically labeled alanine. If the symmetric intermediates rotated freely during the transfer between the enzyme active sites, the ^{13}C label will be equally distributed in the C2 and C3 positions of malate and the intermediates formed from it. When malonate, an inhibitor to succinate dehydrogenase of the Krebs TCA cycle, was added to the labeling mixture, the degree of asymmetrical labeling of alanine was reduced. This decrease in asymmetrical labeling of alanine was greater at higher malonate concentration. At the highest concentration of malonate (500 mM), nonoxidized [3-^{13}C]propionate was also present in the spectrum. Our interpretations and conclusions of these results are as follows: (1) Both succinate and fumarate are transferred between the active sites in a highly orientation-conserved way (substrate channeling). (2) The fraction of orientation-conserved transfer can be reduced by the addition of malonate. This suggests that more symmetrical molecules are dissociating from the enzyme active sites as a result of reduced flux through the TCA cycle and thus more molecules are free to rotate, and distribution of the ^{13}C label in the C2 and C3 positions of the intermediates occurs. (3) There are no major alternative pathways to metabolize propionate in yeast cells since [3-^{13}C]propionate was present in the spectrum at high concentrations of the TCA cycle inhibitor, malonate (500 mM). If important alternative pathways would exist, [3-^{13}C]propionate would have been oxidized through those. The concentration of succinate in heart tissue has been estimated to 50–100 nmol g^{-1} and the maximal rate of oxidation to be less than 5000 nmol min^{-1} g^{-1} (Sumegi *et al.*, 1990). Using these values, the average lifetime of a succinate molecule can be estimated to be about 50/5000 min or greater than 0.5 s. Since the rotational correlation time for free succinate in water is less than 100 ps (Sumegi *et al.*, 1990), the molecule has enough time for about 10^8–10^9 molecular rotations if freely tumbling in water. These calculations also apply for the symmetrical fumarate molecule and together with the ^{13}C NMR results strongly suggest that some kind of organization exists between the enzymes succinate thiokinase, succinate dehydrogenase, and fumarase. These latter interactions have not been demonstrated. However, succinate thiokinase interacts with the α-ketoglutarate dehydrogenase complex, and CS and fumarase have all been shown to interact with MDH [see Srere (1987) for review].

To investigate further the transfer process of the symmetrical intermediates in this part of the TCA cycle, we constructed three new yeast strains consisting of the CS1 mutant yeast cells transformed with plasmids expressing CS1 and the fusion proteins CS1/MDH1 and CS1/HSA, respectively. These transformants contain mitochondrial fusion protein as judged by Western blots on isolated mitochondria, and all have a restored mitochondrial function as judged by the ability to grow an acetate and oxygen respiration tests on isolated coupled mitochondria. The idea was to make a change in another part of the TCA cycle (CS1 position) to see if this can affect the succinate thiokinase–fumarase span of the cycle in a similar way as was seen by the addition of malonate to parental cells. Interestingly, the CS1/HSA genotype did display a changed labeling pattern of alanine when [3-^{13}C]propionate was oxidized. The results show that the fraction of symmetrical intermediates that are transferred with conserved orientation decreases from 86% to 48%. The presence of nonoxidized [3-^{13}C]propionate in the spectrum also indicates that the flux of the TCA cycle is reduced in this transformant, similar to the result seen with high malonate concentration on parental cells. Apparently the CS1/HSA protein is able to affect both the flux and the transfer further down in the pathway, even if it is not directly involved in this part of the energy metabolism itself. These effects were not seen for the CS1 and CS1/MDH1 transformants which behaved like parental yeast. We have no explanation why similar effects are not seen in the other transformants since these also differ significantly from the parental cell. For instance, the CS1 content in the mitochondrial matrix of the CS1 transformant is about 10 times higher than that found in the parental strain. The enzyme level in the CS1/MDH1 transformant is similar to the parental, but in this transformant MDH1 is forced in a fixed position relative to CS1 due to the fusion. Natural interactions have been found between CS and MDH1 (Halper & Srere, 1977). It can therefore be argued that this fusion protein may resemble such a complex and thus does not affect the structure of the putative TCA cycle metabolon as the CS1/HSA fusion does.

Growth experiments on different carbon sources also demonstrated that the CS1/HSA transformant differed from the CS1 and CS1/MDH1 transformant. The doubling time was found to be slightly longer on raffinose. This primarily suggests that the energy metabolism is not as efficient in these constructs since we believe this is the only pathway which has been altered in our experiments. This was also demonstrated by the ^{13}C NMR results, which showed that the TCA cycle was affected. It could be argued that the obtained results depend on the lower CS1 activity found in the CS1/HSA transformant. However, previous reports have demonstrated that the CS1 activity in parental yeast cells is about 30 times higher than the obtained respiratory rate and that the CS1 activity can vary considerably without affecting the oxygen consumption rate (Grigorenko *et al.*, 1990).

The results thus favor the hypothesis that the TCA cycle enzymes may exist as a complex and that proper function of this pathway may be depend on sequential enzyme interactions.

ACKNOWLEDGMENT

The authors thank Dr. L. McAlister-Henn for mutant yeast strains, Virginia Poffenberger for technical assistance, and Penny Kerby for manuscript preparation. Dr. Ravi Ramasamy performed the ^{13}C NMR spectroscopy.

REFERENCES

Barnes, S. J., & Weitzman, P. D. J. (1986) *FEBS Lett. 201*, 267–271.

Beaucage, S. L., & Caruthers, M. H. (1981) *Tetrahedron Lett. 22*, 1859–1862.

Bradford, M. (1976) *Anal. Biochem. 72*, 248–254.

Brent, L. G., & Srere, P. A. (1987) *J. Biol. Chem. 262*, 319–325.

Bülow, L. (1987) *Eur. J. Biochem. 163*, 443–448.

Bülow, L., Ljungcrantz, P., & Mosbach, K. (1985) *Bio/Technology 3*, 821–823.

Clegg, J. S., & Jackson, S. A. (1990) *Arch. Biochem. Biophys. 278*, 452–460.

Datta, A., Merz, J. M., & Spivey, H. O. (1985) *J. Biol. Chem. 260*, 15008–15012.

Daum, G.; Bohni, P. C., & Schatz, G. (1972) *J. Biol. Chem. 257*, 13028–13033.

D'Souza, S. F., & Srere, P. A. (1983) *J. Biol. Chem. 258*, 4706–4709.

Englard, S., & Siegel, L. (1969) *Methods Enzymol. 13*, 99–106.

Fansler, B., & Lowenstein, J. M. (1969) *Methods Enzymol. 13*, 26–30.

Grigorenko, E. V., Small, W. C., Persson, L.-O., & Srere, P. A. (1990) *J. Mol. Recogn. 3*, 215–219.

Hackenbrock, C. R. (1968) *Proc. Natl. Acad. Sci. U.S.A. 61*, 598–605.

Halper, L. A., & Srere, P. A. (1977) *Arch. Biochem. Biophys. 184*, 529–534.

Hill, R. L., & Bradshaw, R. A. (1969) *Methods Enzymol. 13*, 91–99.

Ito, M., Fukada, Y., Murata, K., & Kimura, A. (1983) *J. Bacteriol. 153*, 163–171.

Kâlmân, M., Cserpân, I., Bajszâr, G., Dobi, A., Horvâth, É., Pâzmân, C., & Sinonesits, A. (1990) *Nucleic Acids Res. 18*, 6075–6081.

Kispal, G., Rosenkrantz, M., Guarente, L., & Srere, P. A. (1988) *J. Biol. Chem. 263*, 11145–11149.

Kispal, G., Evans, C. T., Malloy, C., & Srere, P. A. (1989) *J. Biol. Chem. 264*, 11204–11210.

Lindbladh, C., Persson, M., Bülow, L., & Mosbach, K. (1992) *Eur. J. Biochem. 262*, 2786–2790.

Lindbladh, C., Rault, M., Hagglund, C., Small, W. C., Mosbach, K., Bülow, L., Evans, C., & Srere, P. A. (1994) *Biochemistry* (following paper in this issue).

Ljungcrantz, P., Carlsson, H., Månsson, M.-O., Buckel, P., Mosbach, K., & Bülow, L. (1989) *Biochemistry 28*, 8786–8792.

Maniatis, T., Fritsch, E., & Sambrook, J. (1982) *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Messing, J. (1983) *Methods Enzymol. 101*, 20–78.

Minton, A. P. (1990) *Int. J. Biochem. 22*, 1063–1067.

Mosbach, K., & Mattiasson, B. (1970) *Acta Chem. Scand. 24*, 2101–2108.

Niederberger, P., Prasad, R., Miozzari, G., & Kacser, H. (1992) *Biochem. J. 287*, 473–479.

Ohnishi, T., Kawaguchi, K., & Hagihara, B. (1966) *J. Biol. Chem. 241*, 1797–1806.

Plaut, G. W. E. (1969) *Methods Enzymol. 13*, 34–42.

Robinson, J. B., Jr., & Srere, P. A. (1985) *J. Biol. Chem. 260*, 10800–10805.

Robinson, J. B., Jr., Inman, L., Sumegi, B., & Srere, P. A. (1987) *J. Biol. Chem. 262*, 1786–1790.

Schaff, I., Heinisch, J., & Zummermann, F. K. (1989) *Yeast 5*, 285–290.

Shinman, L. M., & Bass, J. P. (1981) *J. Biol. Chem. 256*, 6583–6586.

Spivey, H. O., & Merz, J. M. (1989) *BioEssays 10*, 127–130.

Srere, P. A. (1987) *Annu. Rev. Biochem. 56*, 89–124.

Srere, P. A., & Mosbach, K. (1974) *Annu. Rev. Microbiol. 28*, 61–83.

Srere, P. A., Brazil, M., & Gonen, L. (1963) *Acta Chem. Scand. 17*, S129–S134.

Srere, P. A., Mattiasson, B., & Mosbach, K. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 2534–2538.

Steffan, J. S., Minard, K., & McAlister-Henn, L. (1990) in *Structural and Organizational Aspects of Metabolic Regulation* (Srere, P. A., Jones, M. E., & Mathews, C. K., Eds.) pp 375–403, Wiley-Liss, New York.

Sumegi, B., Sherry, A. D., & Malloy, C. R. (1990) *Biochemistry 29*, 9106–9110.

Sumegi, B., Sherry, A. D., Malloy, C. R., & Srere, P. A. (1993) *Biochemistry 32*, 12725–12729.

Thompson, L. M., Sutherland, P., Steffan, J. S., & McAlister-Henn, L. (1988) *Biochemistry 27*, 8393–8400.

vanJagow, G., & Klingenberg, M. (1970) *Eur. J. Biochem. 12*, 583–592.

Zubay, G. (1983) *Biochemistry*, 1st ed., Addison Wesley Publishing Co. Inc., Reading, MA.