Accelerated Publications

Channeling of TCA Cycle Intermediates in Cultured *Saccharomyces cerevisiae*†

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ABSTRACT: Oxidation of [3-^{13}C]propionate was studied in cultured yeast cells, and the distribution of label in the 2- and 3-positions of alanine was detected by ^{13}C NMR. [3-^{13}C]Propionate forms [2-^{13}C]succinyl-CoA in the mitochondria which then enters the citric acid cycle and forms malate through two symmetrical intermediates, succinate and fumarate. If these symmetrical intermediates randomly diffuse from one enzyme to the next in mitochondria as is normally assumed, then ^{13}C labeling in malate C2 and C3 must be equal. However, any direct transfer of metabolites from site to site between succinate thiokinase, succinate dehydrogenase, and fumarase would result in an uneven distribution of ^{13}C in malate C2 and C3 and any molecules derived from malate. Since pyruvate may be derived from malate via the malic enzyme and subsequently converted into alanine by transamination, any ^{13}C asymmetry in alanine C2 and C3 must directly reflect the ^{13}C distribution in the malate pool. During oxidation of [3-^{13}C]propionate, we detect a significant quantity of labeled alanine, where ^{13}C enrichment in C3 is significantly higher than that in C2. Inhibition of succinate dehydrogenase with malonate or creating conditions that increase the chances of a back-reaction (from malate to fumarate) result in a significant decrease in the asymmetric labeling of alanine. Ubiquinone-deficient yeast cells (having only 10% of the oxidative capacity of wild-type cells) could slowly oxidize propionate, but in this case the ^{13}C labeling was equal in the C2 and C3 of alanine, showing that isotope randomization had occurred. Addition of ubiquinone restores the respiratory activity of this cell line, and the asymmetry in alanine is once again clearly evident. These data show that actively respiring yeast cells can directly transfer intermediates between succinate thiokinase, succinate dehydrogenase, and fumarase under in vivo conditions.

Several laboratories have now reported evidence for organization of mitochondrial enzymes (Beeckmans & Kanarek, 1981; Srere & Sumegi, 1986; Srere, 1985, 1987). This includes evidence for interactions between matrix enzymes (Fahien & Kmiotek, 1983; Halper & Srere, 1977; Hearl & Churchich, 1984; Sumegi & Alkonyi, 1983; Sumegi et al., 1980; Porpaczy et al., 1983; Fahien et al., 1989; Beeckmans & Kanarek, 1981; Beeckmans et al., 1989; Tyiska et al., 1986), evidence for the importance of enzyme–enzyme interactions in vivo in mutant yeast cells (Kispal et al., 1989), and evidence for interactions between matrix enzymes and inner mitochondrial membrane proteins (Sumegi & Srere, 1984a,b; Kispal et al., 1986; D'Souza & Srere, 1983). It has also been shown in some systems that interactions between mitochondrial enzymes have a positive kinetic effect (Sumegi et al., 1980; Porpaczy et al., 1983; Tompa et al., 1987), and channeling of intermediates was demonstrated in one in vitro system (Fukushima et al., 1989). Even in the most physiological investigations, the cells or organelles were invariably permeabilized (Robinson et al., 1987; Cohen et al., 1987), and this process could have altered local enzyme environments enough to produce a kinetic difference. However, since there are now in vitro data (Srivastava & Bernhard, 1986; Srere & Sumegi, 1986; Srere, 1987) for enzyme–enzyme interactions in several metabolic pathways, it remains possible that direct evidence for the catalytic consequences of enzyme–enzyme interactions might be obtained by carefully designed experiments on an intact cell system. It might be possible, for example, to detect channeling between TCA cycle enzymes in intact cells if one could generate asymmetrically labeled succinyl-CoA. Since malate formed from succinyl-CoA must go through two symmetrical intermediates, succinate and fumarate, free diffusion of these intermediates in the mitochondrial matrix would ensure that labeling in malate would be symmetrical. This is a generally accepted concept by readers of all modern biochemistry texts (Stryer, 1988; Zubay, 1988). If, however, the intermediates were channeled between succinyl thiokinase, succinate dehydrogenase, and fumarase, then asymmetric labeling would be maintained in any malate formed along this pathway. Early experiments using radiolabeled intermediates provided no evidence for channeling (Ehrensward et al., 1951; Ajl & Kamen, 1951; Lee & Lifson, 1951; Mahler & Cordes, 1971), although measures were not always taken to avoid back-reactions and experimental precision was considerably worse than present conditions allow. More recently, there was an attempt to determine whether succinate and fumarate can freely rotate during oxidation of [5-^{13}C]glutamate in intact liver mitochondria (Bernhard & Tompa, 1990). The results of those experiments supported the generally accepted concept that fumarate and succinate freely rotate in intact mitochondria. However, we felt that

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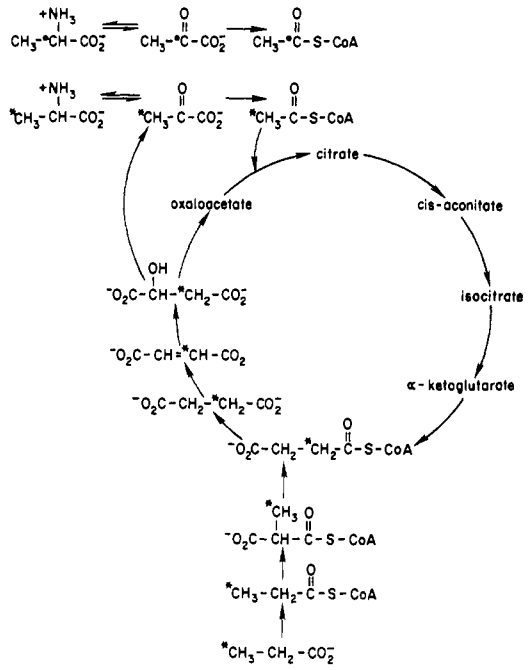

FIGURE 1: Propionate oxidation pathway. [3-^{13}C]Propionate is transformed into [2-^{13}C]succinyl-CoA in mitochondria where it enters the citric acid cycle. An asterisk shows the fate of ^{13}C directly transferred between [2-^{13}C]succinyl-CoA and malate without rotation and a solid circle shows the fate of ^{13}C if one of the symmetric intermediates undergoes a 180° rotation.

their experimental conditions (high mitochondrial density that might stimulate release and uptake of intermediates and the use of H_2O_2) could have led to a significant change in mitochondrial structure, so we designed further experiments using cultured yeast cells to test for possible restriction of rotation of TCA cycle intermediates under in vivo conditions.

Asymmetric labeling of four carbon atom intermediates was achieved by giving [3-^{13}C]propionate to the yeast cells. [3-^{13}C]Propionate is activated to propionyl-CoA and then converted to methylmalonyl-CoA, followed by formation of [2-^{13}C]succinyl-CoA (Stryer, 1988), before being further oxidized in the TCA cycle (Figure 1). In the absence of tight channeling between TCA cycle enzymes, the ^{13}C should become evenly distributed in either C2 or C3 of malate. However, if channeling between TCA cycle enzymes occurs in intact yeast cells, malate and any intermediate derived from malate (for example, alanine) must be labeled asymmetrically in C2 and C3. The results of our experiments in respiratory-competent yeast cells provided the first evidence that substrate channeling between TCA cycle enzymes does indeed occur in vivo.

Materials and Methods

Materials. Sodium [3-^{13}C]propionate (98.7% ^{13}C) was obtained from MDS Isotopes. Cytochrome *c*, ubiquinone$_0$, ubiquinone$_6$, and ubiquinone$_{10}$ were purchased from Sigma. All other chemicals were of the highest purity commercially available.

Strains and Culture Conditions. N5-91 ubiquinone-deficient yeast [described in Tzagoloff (1975)] was isogenic with the wild-type strain D 273-10B. Both strains were a generous gift from Dr. Alexander Tzagoloff. The cells were grown in standard YP medium with 0.25% glucose and 2% galactose as carbon sources. Cells were harvested in the late log phase by centrifugation at 4000 rpm for 10 min.

Propionate Oxidation. Two grams of packed yeast cells, either D 273-10B or N5-91, was resuspended in 15 mL of minimal medium (pH = 6.2) containing 3 mg of [3-^{13}C]-

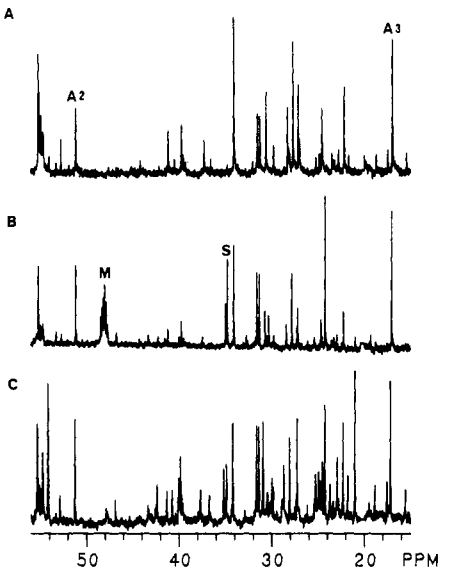

FIGURE 2: ^{13}C NMR spectra of yeast cell extracts. Yeast cells were grown in rich medium containing 2% galactose and 0.25% glucose. In the late log phase cells were harvested and 2 g of cell pellets was resuspended in 15 mL of yeast minimal medium (pH balanced at 6.2) containing 3 mg of [3-^{13}C]propionate in all cases. (A) The medium contained [3-^{13}C]propionate only; (B) the medium also contained 50 mM malonate; (C) the medium also contained 30 mg of glycerol. The resonances labeled A2, A3, S, and M (natural abundance) refer to the C2 and C3 of alanine, the C2=C3 of succinate, and the C2 of malonate, respectively. In (A) and (B), the reaction mixtures were well supplied with O_2 while in (C) the gas phase above the reaction mixture was exchanged with N_2 gas to limit the oxygen supply. All reactions was stopped by the addition of 4% perchloric acid, and samples were prepared for NMR spectroscopy as described under Materials and Methods.

propionate (sodium salt). The resuspended yeast cells were incubated at 30 °C and were shaken vigorously to supply the required amount of oxygen. When required, the oxygen supply was limited by placing the reaction mixture in a smaller, closed tube after the air in the tube had been exchanged with N_2. Incubation usually lasted for 30–60 min. The reaction was stopped by adding perchloric acid to a final concentration of 4%, the supernatant was neutralized and freeze-dried, and the resulting powder was redissolved in 0.8 mL of D_2O for NMR studies.

NMR Spectroscopy. High-resolution ^{13}C NMR spectra were recorded on a GN-500 spectrometer at 11.75 T. The spin–lattice relaxation times of the C2 and C3 resonances of alanine were found to be 3.2 and 1.7 s, respectively, under solution conditions approximately equal to those of the extracts. All spectra reported in this work were acquired by using a 45° carbon pulse and a 6-s delay between pulses to ensure non-saturating conditions. All samples were maintained at 25 °C during data acquisition.

Results

The oxidation of [3-^{13}C]propionate by wild-type yeast cells (D 273-10B) was studied in vivo in pH-balanced minimal medium. A significant difference between the ^{13}C enrichment levels in the C2 and C3 of alanine was clearly evident in the ^{13}C NMR spectra of the extracts of these cells (Figure 2). This same experiment was performed with [2-^{13}C]propionate (data not shown) and the opposite enrichment pattern was observed (C2 higher than C3). These spectra were acquired under conditions that ensure equivalent peak intensities for all protonated carbon resonances within any given metabolite. If there had been free diffusion of TCA cycle intermediates in the yeast mitochondria, ^{13}C originating in [3-^{13}C]propionate

Table I: ^{13}C Enrichment of Alanine during [3-^{13}C]Propionate Oxidation in *Saccharomyces cerevisiae*[a]

strains and exptl conditions	areas of alanine resonances[b] carbon 2	carbon 3	C3/C2 ratio[c]
D 273-10B	1904	4487	2.35
D 273-10B + malonate	1269	1669	1.31
D 273-10B + glycerol	2417	3254	1.34
N5-91	1289	1337	1.03
N5-91 + ubiquinone$_0$	1292	2357	1.82

[a] All experimental conditions were as described in Figures 2 and 3. [b] Values are given in arbitrary units. Peak areas were determined by a line-fit analysis using the GE software; errors in peak areas were less then 2%. [c] These values are representative of four to five different experiments, each of which shows the same trends as indicated here.

should have equally labeled the C2 and C3 positions of malate and oxaloacetate and the corresponding position of any metabolite derived from these intermediates, such as pyruvate or alanine. Unequal distribution in these intermediates would be possible only if the succinate and fumarate formed from [2-^{13}C]succinyl-CoA were directly transferred between succinate thiokinase, succinate dehydrogenase, and fumarase. As the quantitative data show (Table I), there is 2.35 times more ^{13}C in alanine C3 than in alanine C2. The experiment demonstrated in Figure 2A was repeated five times and in all cases the C3/C2 ratio was between 2 and 4.

When oxidation of [3-^{13}C]propionate was carried out in the presence of 50 mM malonate, there was still some difference in the levels of ^{13}C in alanine C2 and C3, but the ratio decreased to 1.31 (Table I). Since malonate inhibition of succinate dehydrogenase results in a significant increase in the succinate concentration (^{13}C-enriched succinate is visible in the spectrum, Figure 2B), a large pool of succinate is now available to exchange with those succinate molecules associated with the active sites of succinate thiokinase and succinate dehydrogenase, and this increases the chances for randomization of the ^{13}C label. The fact that alanine still contained a somewhat higher ^{13}C enrichment in C3 than C2 indicates that substrate channeling can be detected even in the presence of excess succinate.

[3-^{13}C]Propionate oxidation by yeast was also studied in the presence of glycerol under conditions with reduced oxygen availability (Figure 2C, Table I). As these experimental conditions should favor the back-reactions in the fumarase and perhaps succinate dehydrogenase reactions, we expected to find equal ^{13}C enrichment at the two alanine carbons. The experimental data show that enrichment of alanine C2 and C3 is now very nearly the same (1.34), indicating once again that a significant randomization of ^{13}C had occurred in these reactions in the TCA cycle.

Oxidation of propionate was also studied in a ubiquinone-deficient yeast cell line (N5-91), which showed only 5–10% of the respiratory activity of the respective wild-type cell line (D273-10B). In this case, ^{13}C enrichment in alanine C2 and C3 was identical (1.03; see Table I), suggesting that the decrease in respiration decreases the flux through the TCA cycle (likely at the succinate dehydrogenase step), thereby increasing the probability that a succinate molecule will mix with other succinate pools and hence randomize before proceeding to fumarate. This cell line regains its respiratory activity upon addition of ubiquinone, making it possible to further evaluate the relationship between respiration and substrate channeling. The concentration of ubiquinone necessary to restore the NADH:cytochrome *c* oxidoreductase activity of N5-91 was determined in an in vitro assay, as shown in Table II. The N5-91 yeast cell line was grown as described, and [3-^{13}C]-

Table II: Effect of Added Ubiquinone Derivatives on the Activity of NADH:Ubiquinone Oxidoreductase Activity in Ubiquinone-Deficient Yeast Cells[a]

addition of ubiquinone derivatives	NADH:ubiquinone oxidoreductase activity[b] N5-91	D 273-10B
none	8 ± 2	100 ± 8
ubiquinone$_0$		
3 μg/mL	25 ± 3	nd[c]
6 μg/mL	41 ± 4	nd
10 μg/mL	61 ± 6	nd
20 μg/mL	96 ± 7	106 ± 9
ubiquinone$_6$		
20 μg/mL	24 ± 3	108 ± 7

[a] Both wild-type and ubiquinone-deficient cell lines were grown under the same conditions in rich media containing 2% galactose and 0.25% glucose. Cells were harvested in the late log phase and spheroplasts were prepared with lyticase (Hauswirth et al., 1987). Spheroplasts were disrupted in a Potter-Elvehjem homogenizer, and the mitochondria were separated by centrifugation as described (Hauswirth et al., 1987). NADH:cytochrome *c* oxidoreductase activity was measured as described previously (Hatefi, 1976) except that ubiquinone$_0$ or ubiquinone$_6$ was used. [b] Expressed as percent of wild-type yeast activity (mean ± SD for three experiments). [c] nd = not determined.

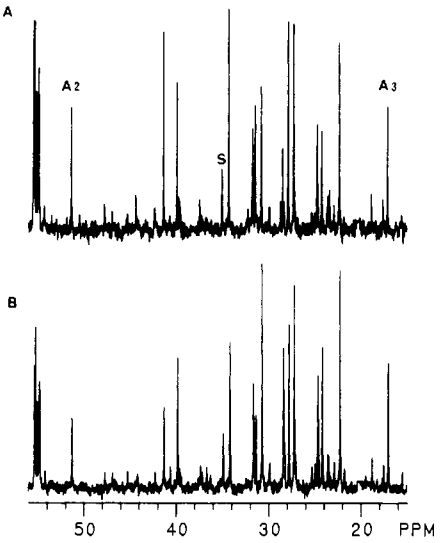

FIGURE 3: ^{13}C NMR spectra of ubiquinone-deficient (N5-91) yeast cell extracts after oxidation of [3-^{13}C]propionate. Experimental conditions were the same as in Figure 2A. (A) Spectrum of ubiquinone-deficient yeast and (B) same yeast with 0.02 mg/mL ubiquinone$_0$ added back to the reaction mixture. The resonance designations are the same as those described in the legend to Figure 2.

propionate oxidation was studied under the same conditions except that respiration was restored by the addition of 0.02 mg of ubiquinone$_0$/mL of reaction mixture. Under these experimental conditions, the alanine derived from [3-^{13}C]-propionate had amuch higher ^{13}C enrichment in C3 than in C2 (1.83) (Figure 3B and Table I). This suggests that active respiration is a prerequisite for substrate channeling between these TCA cycle enzymes.

Discussion

Specific interactions between sequential mitochondrial enzymes have now been demonstrated for several systems and by multiple investigators. It has also been shown that enzyme–enzyme interactions influence the catalytic efficiency of sequential reactions (Sumegi et al., 1980; Porpaczy et al., 1983; Fukushima et al., 1989; Tompa et al., 1987; Robinson et al., 1987). Although there are only a limited number of kinetic studies on interacting mitochondrial enzymes, the

available data indicate that mitochondrial enzyme–enzyme interactions may be an important factor in regulation of mitochondrial metabolism (Srere, 1987). Since most kinetic studies were performed under in vitro conditions, the question of whether these same kinetic advantages also apply in vivo remained. Although there have been studies on permeabilized mitochondria (Robinson et al., 1987; Sumegi & Porpaczy, 1989) and cells (Cohen et al., 1987) that support the kinetic importance of organization in vivo, there is no definitive evidence for substrate channeling in intact systems. Our data on oxidation of [3-^{13}C]propionate in intact yeast cells show for the first time that asymmetrically labeled [2-^{13}C]-succinyl-CoA can be transformed into asymmetrically labeled alanine, presumably through asymmetrically labeled oxaloacetate and malate. This occurs even though malate is formed from succinyl-CoA through two symmetric intermediates (succinate and fumarate). If these intermediates were able to rotate during their transfer from one enzyme active site to the next, those carbon atoms containing enriched ^{13}C would have become evenly distributed between the C2 and C3 positions of malate and in all intermediates formed from malate, such as alanine (Figure 1). The rotational correlation time for free succinate, or fumarate, in water is less than 100 ps (Akitt, 1987). Since the concentration of succinate in heart tissue, for example, is about 50–100 nmol/g wet weight (Bergmeyer, 1974) and the maximal rate of succinate oxidation is less than 5000 nmol (g wet weight)$^{-1}$ min^{-1} (Bergmeyer, 1974), one can estimate that the average lifetime of a succinate molecule is roughly 50/5000 min or greater than 0.5 s. This is sufficient time for about 10^9 molecular rotations through 180° for a freely tumbling succinate molecule in water. This calculation can also be carried out for yeast cells under our experimental conditions, and the lifetime of succinate is approximately the same order of magnitude. In addition, fumarate is also a symmetric molecule that could rotate 10^8–10^9 times before being transformed into malate. Therefore, in the absence of organization and substrate channeling, we should not be able to detect any asymmetry in malate or other intermediates derived from malate. The fact that there is very significant asymmetry in the labeling of alanine (Table I) shows unequivocally that intermediates in the succinyl-CoA to malate span of the TCA cycle cannot be randomly diffusing. The asymmetric labeling data can only be interpreted by proposing that these intermediates are directly transferred between sequential enzymes. Furthermore, one may conclude from these data that the molecules are transferred between enzymes with a specific orientation; i.e., the C2 of succinyl-CoA (methylene carbon nearest the thioester linkage) becomes the C3 carbon of malate (methylene carbon). These results also show that fumarate bound in the active site of fumarase has a defined orientation such that only one of the methylene carbons (that derived from the C3 of succinyl-CoA) can accept a OH group from water.

There is no experimental evidence for direct physical interactions between succinyl thiokinase, succinate dehydrogenase, and/or fumarase, but there is evidence to suggest that succinyl thiokinase is indirectly (via the α-ketoglutarate dehydrogenase complex) bound to complex I (Porpaczy et al., 1987). In addition, there are experimental data suggesting an association between complex I and complex II (Ragen & Heron, 1978; Yu et al., 1974), and we have previously obtained in vitro data which suggest that (NAD) isocitrate dehydrogenase, the α-ketoglutarate dehydrogenase complex, and succinyl thiokinase are in the immediate microenvironment of complexes I and II (complex II contains succinate dehydrogenase) facilitating direct metabolite transfer (Porpaczy et al., 1987). The new NMR data reported here demonstrate unequivocally that direct transfer of intermediates occurs in vivo, supporting the many in vitro results showing spatial organization of TCA cycle enzymes.

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Articles

A Molecular Mechanical Force Field for the Conformational Analysis of Oligosaccharides: Comparison of Theoretical and Crystal Structures of Manα1–3Manβ1–4GlcNAc†

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ABSTRACT: A molecular mechanical force field is described for the conformational analysis of oligosaccharides. This force field has been derived by the addition of new parameters to the AMBER force field and is compatible with simulations of proteins. This new parametrization is assessed by comparison of the theoretically predicted conformations of Manα1–3Manβ1–4GlcNAc with the corresponding crystal structure. Molecular dynamics simulation data are presented for this structure both in vacuo and with the explicit inclusion of water molecules. While the former demonstrate significant torsional oscillations about glycosidic linkages at physiological temperature, in the latter these oscillations are highly damped due to the stabilizing influence of a "cage" of solvent–solvent and solvent–solute hydrogen bonds.

The primary tool for the conformational analysis of macromolecules in solution is the ^{1}H NMR nuclear Overhauser effect (NOE) (Noggle & Schirmer, 1971; Neuhaus & Williamson, 1989). By virtue of the inverse sixth power dependence of this parameter upon internuclear distance (r), a highly sensitive conformational "ruler" is available when r is small (<5 Å). However, this function carries with it an inherent penalty when r varies with time, as, for example, in the presence of internal motion. In these cases NOE measurements can suggest a dominant conformer that is completely erroneous since the measured value of the NOE may be heavily weighted in favor of conformers where r is small. Nevertheless, it has been adequately demonstrated that NOE measurements in proteins and nucleic acids can give an average solution structure which compares very favorably with that derived by X-ray diffraction studies (Wuthrich, 1986, and references cited therein). The primary reason for the success of the NOE method lies in the very large number of available distance constraints: when analyzed with distance matrix algorithms in Cartesian or dihedral angle space, the rms atomic deviations at least for the C_α backbone in proteins are sufficiently small for a series of random starting structures that an average solution structure can be defined with confidence (Wuthrich, 1986, and references cited therein). This structure may then be refined by use of energy minimization and molecular dynamics algorithms together with an appropriate molecular mechanical force field (Clore et al., 1986).

In principle, the solution conformations of oligosaccharides can be derived in a manner similar to that for proteins and nucleic acids. However, the above approach has not been adopted for at least two reasons. First, the number of distance constraints available from NOE measurements is very much smaller than in proteins or nucleic acids. Often only one or two NOEs are available across glycosidic linkages, and generally, no NOEs between distance regions of the molecule are observed. Very poor convergence would thus be obtained in distance geometry calculations. Second, a suitable force field for the conformational analysis of oligosaccharides has not been available. Since NOE data in oligosaccharides may not provide sufficient constraints when used alone, the availability

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