The enzymes of the tricarboxylic acid cycle in the mitochondrial matrix are proposed to form a multienzyme complex, in which there is channeling of substrates between enzyme active sites. However, no direct evidence has been obtained in vivo for the involvement of these enzymes in such a complex. We have labeled the tricarboxylic acid cycle enzyme, citrate synthase 1, in the yeast Saccharomyces cerevisiae, by biosynthetic incorporation of 5-fluorotryptophan. Comparison of the 19F NMR resonance intensities from the labeled enzyme in the intact cell and in cell-free lysates indicated that the enzyme is motionally restricted in vivo, consistent with its participation in a multienzyme complex.

The protein concentration in the mitochondrial matrix is thought to be very high, between 270 and 560 mg/ml depending on the functional state of the organelle (1, 2). This very high protein concentration has been proposed to restrict the diffusion of enzymes and metabolites and to promote the association of matrix enzymes. This has led to the suggestion that the reactions of the tricarboxylic acid cycle may occur via channeling of metabolites between enzyme active sites in a multienzyme complex (3–6) or metabolon (7).

There is a large body of data that supports the idea of a tricarboxylic acid cycle metabolon. Specific associations have been demonstrated in vitro between tricarboxylic acid cycle enzymes and also between these enzymes and components of the respiratory chain and other proteins in the inner mitochondrial membrane (3). Complexes able to catalyze several consecutive steps of the tricarboxylic acid cycle have been isolated following gentle disruption of both liver mitochondria (8, 9) and a number of different microorganisms (10, 11). Coupled reactions within complexes of tricarboxylic acid cycle enzymes have been shown to have a kinetic advantage when compared with the completely solubilized systems (3, 9). Molecular modeling studies on a citrate synthase-malate dehydrogenase fusion protein, for which there was experimental evidence of substrate channeling (12), showed that there could be a very efficient, electrostatically based, channeling mechanism for substrate transfer between the enzymes active sites (13). Evidence has also been obtained in vivo for a tricarboxylic acid cycle enzyme complex. Disruptions of the genes for citrate synthase and malate dehydrogenase in yeast resulted in cells that were unable to grow on acetate (14). This was despite the fact that there are isozymes in the cytosol which could, in principle, bypass the resulting blocks in the cycle. In the case of citrate synthase, introduction of a structurally similar but catalytically inactive mutant resulted in restoration of tricarboxylic acid cycle function and growth on acetate (15). These studies have been interpreted as indicating the presence of a complex of tricarboxylic acid cycle enzymes in which the enzymes have structural as well as catalytic roles. There is also evidence for substrate channeling in vivo. Analysis of 13C labeling patterns in metabolites derived from tricarboxylic acid cycle intermediates indicated channeling of succinate and fumarate in the cycle (16, 17).

Demonstration of these weak enzyme complexes in situ has been difficult, however, because many of them are dissociated during isolation due to dilution effects. There is also no direct evidence for their presence in an intact cell. We show here, using NMR measurements on a minimally derivatized enzyme of the tricarboxylic acid cycle, that it exists in a motionally restricted form in the yeast mitochondrial matrix in vivo, consistent with its participation in a multienzyme complex.

EXPERIMENTAL PROCEDURES

Materials—Saccharomyces cerevisiae strain BJ2168 (α, gal2, ura3–52, leu2–3, leu2–112, trp1–, pep4–, prb1–1122, pre1–407) was used in these studies (18). Growth media were obtained from Difco. Oligonucleotide linkers were supplied by New England Biolabs. All other reagents were obtained from Sigma or from Boehringer Mannheim. Protein concentrations were determined with a dye binding assay (19) kit from Bio-Rad, with bovine serum albumin as a standard.

Plasmid Construction—The coding sequence for yeast mitochondrial citrate synthase 1 (CIT1) (20) was cloned into the EcoRI–HindIII fragment containing the plasmid pYES2.0 (Invitrogen Corp.) to generate the plasmid pYES2.0-CIT1. This plasmid was linearized by digestion with BamHI and the 1.5-kilobase pair fragment containing the CIT1 coding sequence was ligated into the unique BgIII expression site of the LEU2-expressing plasmid pK49V (21). These manipulations generated the plasmid pBF208, which expressed yeast mitochondrial citrate synthase 1 (CIT1, EC 4.1.3.7) under the control of a galactose-inducible version of the yeast phosphoglycerate kinase promoter.

Enzyme Labeling—Cells were transformed with the plasmid pBF208 by the method of Hinnen et al. (22). CIT1 was fluorine-labeled by inducing enzyme expression in stationary phase cells in the presence of 5-fluoro-L-tryptophan (5-FTrp).1 The labeling protocol employed was similar to that used previously to label phosphoglycerate kinase (23). Briefly, 2.5 × 10^5 cells were used to inoculate a 500-mL culture containing 2% glucose, 2% lactate, and 1% yeast extract. This culture was grown for 24 h, by which time the cells were in stationary phase at a density of approximately 2 × 10^9 cells/mL. The cells were washed and resuspended in 500 ml of medium containing 2% galactose, 0.67% yeast nitrogen base, and a mixture of amino acids lacking tryptophan and leucine. After 2 h, 50 ml of a 0.2% solution of 5-FTrp was added and the culture incubated for a further 24 h prior to cell harvesting.

Enzyme Assay—Cells were disrupted by vigorous agitation in extrac-

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1 The abbreviations used are: 5-FTrp, 5-fluoro-L-tryptophan; CIT1, yeast mitochondrial citrate synthase 1; CIT2, yeast peroxisomal citrate synthase 2; CIT3, yeast mitochondrial citrate synthase 3; GFP, green fluorescent protein.
tion buffer (50 mM sodium phosphate, 5 mM EDTA, 1% Triton X-100, pH 7.0). Citrate synthase activity was assayed as described in (24). Enzyme activities are expressed per milliliter of cell water, assuming that 1.67 g of cells contain 1 ml of cell water (25). All means are quoted with their S.E.

Cell Immobilization and Perfusion—Cells were immobilized and perfused as described previously (26). The cells (6 g wet weight with 6 ml of 1.8% agarose) were perfused with an oxygenated buffer that had the same composition as that used for protein labeling, except that it was supplemented with 0.002% tryptophan instead of 5-FTrp.

Protein Purification—Mitochondria were isolated as described in Ref. 27. All procedures were performed at 4 °C. Isolated mitochondria were disrupted by sonication, after the addition of Triton X-100 to 0.01% (v/v), and the debris and unlysed mitochondria removed by centrifugation (10 min, 15,000 × g). The extract was brought to 85% saturation with (NH₄)₂SO₄ and stirred for 30 min before centrifugation for 30 min at 27,000 × g. The resulting pellet was dissolved in a buffer containing 1.7 M (NH₄)₂SO₄, 50 mM Tris-HCl, 5 mM EDTA, and 2 mM diithothreitol, pH 8.0, and applied to a column of octyl-Sepharose (Amersham Pharmacia Biotech) pre-equilibrated with the same buffer. Citrate synthase was eluted from the column with a linear (NH₄)₂SO₄ gradient ranging from 1.7 to 0 M. Fraction purity was assessed by SDS-polyacrylamide gel electrophoresis. Fractions that contained essentially pure CIT1 were pooled and concentrated using an Amicon stirred ultrafiltration cell (YM30 membrane) until the enzyme reached a molecular mass of greater than 1 mg/ml. CIT1 was stored at 4 °C as an (NH₄)₂SO₄ precipitate.

For NMR measurements the purified enzyme was desalted by gel filtration, using NAP-5 columns (Amersham Pharmacia Biotech), into NMR buffer (50 mM HEPES, 130 mM potassium acetate, and 2 mM diithothreitol, pH 7.2) and then concentrated using Amicon centricon 10 microconcentrators. Samples contained 10% v/v 2H₂O for a field frequency lock. pH measurements were not corrected for any deuterium isotope effect. Sucrose was added to give solutions of higher viscosity where required, and the final protein concentration was typically 15–25 mg/ml.

NMR Measurements—NMR experiments were performed at a 19F resonance frequency of 376.29 MHz, as described previously (26). Fluorine-19 chemical shifts are quoted relative to p-fluorophenylalanine standards, either a 100 µM internal standard in solutions of the purified enzyme or an external standard contained in a coaxial capillary with aqueous viscosity, was 1.1 ± 0.2 s, in agreement with the value expected from theory of 1.0 s. The T₁ of the envelope of resonances (Fig. 1, resonances 2–6) was also near to 1 s, at 1.1 ± 0.1 s. These data indicate that the tryptophans are relatively immobile and have a correlation time similar to that of the whole protein. The location of the tryptophans within the protein structure is consistent with this apparent lack of mobility.

RESULTS

Protein Labeling—A mitochondrial isoform of citrate synthase (CIT1) was selectively fluorine-labeled in vivo by induc-
in the cells during this period. The $^{19}$F NMR data were ac-
mements. There was no significant loss of citrate synthase activity
bols show experimentally measured
The sym-
resonances from CIT1 on the viscosity of the medium.
A
(26).

Mapping of the CIT1 sequence onto the structure of the bovine
heart and chicken heart enzymes and investigation of the sol-
vent accessibility of the tryptophan residues, with the pro-
grams MODELLER and NACCESS (36, 37), indicate that all
are buried or constrained within the protein structure. The $T_1$
of the envelope of resonances increased with solvent viscosity
(Fig. 2), and this increase showed reasonable agreement with
theoretically calculated values (26). The line widths also
showed good agreement with theory. The line width of reso-
nance 1 (Fig. 1) was measured at 60 Hz in a solution of near-
aqueous viscosity, and the calculated value was 52 Hz.

$^{19}$F NMR Measurements in Vivo—Spectra were obtained
from cells that had been immobilized in agarose gel threads
and maintained in a metabolic steady state, during NMR data
acquisition, by perifusion with oxygenated medium (Fig. 3).
The metabolic status of the cells was confirmed by $^{13}$C NMR
spectra (38) acquired before and after the $^{19}$F NMR exper-
iments. There was no significant loss of citrate synthase activity
in the cells during this period. The $^{19}$F NMR data were ac-
tured in a solution of near-aqueous viscosity i.e. approximately 1 s.

Unresolved $^{19}$F resonances were observed in cells that had
been induced to express CIT1 in the presence of 5-FTrp (Fig.
3C). However similar resonances were also observed in con-
tral cells, when expressed on a cell water basis, was $285 \pm 45$
$\mu M$ (mean $\pm$ S.E., $n = 3$) (Fig. 3B), showing that these
resonances were due predominantly to nonspecific incorporation of
5-FTrp into cell proteins and that these proteins were fully
visible in the $^{19}$F spectra of the cells. However spectra of dia-
lyzed lysates prepared from cells overexpressing CIT1 (Fig. 3D)
were very similar to those obtained from the purified enzyme
(Fig. 1), in particular the resolved downfield resonance (reso-
nance 1, Fig. 1), which was never observed in spectra of cells,
was clearly visible. Furthermore the concentration of detecta-
table fluorine in these lysates, expressed on a cell water basis,
was much higher at $850 \pm 110 \mu M$ (mean $\pm$ S.E., $n = 3$). Thus
the labeled enzyme, which was undetectable in $^{19}$F spectra of
the cells, was visible in the diluted extracts prepared from
these same cells.

DISCUSSION

There is a considerable body of evidence for the organization
of the enzymes of the tricarboxylic acid cycle in a multienzyme
complex, in which there is channeling of cycle intermediates
(reviewed in (3, 4)). The concept of a tricarboxylic acid cycle
metabolon, however, has remained controversial as it has been
difficult to isolate an intact complex from the cell. The rela-
tively weak interactions between the enzymes, which are fa-
vored in the cell by the very high protein concentrations in the
mitochondrial matrix (39, 40), are disrupted by the dilution
that occurs during cell extraction.

In this study we have investigated the rotational mobility of
a tricarboxylic acid cycle enzyme, CIT1, in the matrix of yeast
mitochondria in vivo using $^{19}$F NMR measurements on a fluo-
rotyptophan resonances from the labeled enzyme in media of specified
values of the resonances are expected to be linearly

dashed line

FIG. 2. Dependence of the $T_1$ values of the 5-fluorotryptophan
resonances from CIT1 on the viscosity of the medium. The sym-
ples were obtained by linear regression. The dashed line
shows the expected variation of $T_1$ values of the resonances are similar to those of the

nucleases in yeast. Phosphoglycerate kinase and hexokinase
were found to be tumbling in a cytoplasm with a viscosity
approximately twice that of water (26), in good agreement with
fluorescence measurements of cytoplasmic viscosity in mam-
alian cells (see for example (41, 42)). Pyruvate kinase, how-
ever, yielded no detectable NMR signals in vivo, indicating that
there was some degree of motional restriction of this enzyme in
the cell. This was thought to be due to binding of the enzyme to
other cellular macromolecules.

CIT1, like pyruvate kinase, showed no detectable fluorine
resonances in the intact cell, although it was readily detectable
in diluted cell extracts. The $^{19}$F signals that were observed in
the cell could be assigned to nonspecific labeling of cell proteins
(see Fig. 3). There are several possible explanations for this
lack of NMR visibility of CIT1 in the cell.

NMR and fluorescence measurements have indicated a mi-

tochondrial matrix viscosity of between 25 and 37 times that of
water (43, 44). Such a high viscosity would lead to substantial
broadening of the $^{19}$F resonances of labeled CIT1 and an in-
crease in their $T_1$ relaxation times, resulting in signal satura-
tion under the NMR acquisition conditions employed. For ex-
ample at a viscosity of 25 centipoise (centipoise = $10^{-2}$ poise),
the calculated line width of the $^{19}$F resonances would be 1200
Hz and the $T_1$ 22 s. With this $T_1$, the signals detected in the cell
would be substantially saturated, and their intensities would
be only 26% of the fully relaxed value. However recent time
resolved fluorescence measurements on mitochondrial tar-
gated GFP (45) showed the matrix viscosity to be close to that
of an aqueous solution. The high apparent viscosity estimated from the earlier fluorescence anisotropy measurements (43)
was shown to be explicable by probe binding.

The lack of NMR visibility of labeled CIT1 in vivo could be
due to broadening of its resonances by paramagnetic ions pre-
sent in the mitochondrial matrix. This, however, seems unlikely
as the tryptophan residues are at least partially buried within
the protein and should thus be inaccessible to paramagnetic ions. Furthermore there is no evidence for paramagnetic ions significantly affecting the relaxation rates of resonances from intramitochondrial metabolites, including the \(^1\)H resonances of ATP (46–49) and inorganic phosphate (48) and the \(^1\)H resonance of water (44).

The most likely explanation, therefore, for the NMR invisibility of CIT1 is that its \(^1\)H resonances are broadened by an increase in its correlation time, due to binding to other matrix proteins. A complex containing five tricarboxylic acid cycle enzymes has been isolated (8–11). Assuming that there is one molecule of each enzyme in the complex then this would have a molecular mass of approximately 600 kDa. If the complex behaves as a hard sphere and CIT1 has the same correlation time as the whole complex, then the line width of the fluoride resonances of CIT1 would be increased to 210 Hz. Even with this degree of line broadening the protein could still be detectable in the cell. However since we have overexpressed the enzyme by a factor of 10, it is unlikely that much of the labeled enzyme could participate in such a stoichiometric complex. The enzyme has been isolated (8–11). Assuming that there is one molecule of each enzyme in the complex then this would have a molecular mass of approximately 600 kDa. If the complex behaves as a hard sphere and CIT1 has the same correlation time as the whole complex, then the line width of the fluoride resonances of CIT1 would be increased to 210 Hz. Even with this degree of line broadening the protein could still be detectable in the cell. However since we have overexpressed the enzyme by a factor of 10, it is unlikely that much of the labeled enzyme could participate in such a stoichiometric complex. The enzyme has also been shown to bind, with other tricarboxylic acid cycle enzymes, to the mitochondrial membrane (3, 4, 14). This would lead to a much larger increase in the enzyme’s correlation time and thus the \(^1\)H resonance line widths. Under these circumstances the NMR signals would be broadened beyond detection in vivo and therefore membrane binding is a much better candidate as an explanation for the invisibility of CIT1. Immobilization of a GFP-tagged matrix enzyme was observed recently by Partikian et al. (45). By contrast, fluorescence recovery after photobleaching measurements on free GFP showed that its diffusion was relatively rapid, being only three to four times slower than in water (45). In order to explain the rapid diffusion of GFP it was proposed that the matrix proteins are organized peripherally in membrane-associated complexes, thus creating a central aqueous region with relatively low protein density and low viscosity. Such a domain would allow the rapid and unrestricted diffusion of solutes. This model is consistent with the data presented here demonstrating immobilization of CIT1 in vivo and with previous studies showing that it binds with other tricarboxylic acid cycle enzymes to the mitochondrial membrane.

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REFERENCES
1. Hackenbrock, C. R. (1968) Proc. Natl. Acad. Sci. U. S. A. 61, 598–605
2. Srere, P. A. (1980) Trends Biochem. Sci. 5, 120–121
3. Srere, P. A. (1987) Annu. Rev. Biochem. 56, 89–124
4. Srere, P. A. (1993) Biol. Chem. 374, 833–842
5. Westerhoff, H. V., and Welch, G. R. (1992) Curr. Top. Cell. Regul. 33, 361–390
6. Welch, G. R., and Easterby, J. S. (1994) Trends Biochem. Sci. 19, 193–197
7. Srere, P. A. (1985) Trends Biochem. Sci. 10, 109–110
8. Robinson, J. B., and Srere, P. A. (1985) J. Biol. Chem. 260, 10860–10865
9. Robinson, J. B., Inman, L., Sumegi, B., and Srere, P. A. (1987) J. Biol. Chem. 262, 1768–1770
10. Barnes, S. J., and Weitzman, P. D. J. (1986) FEBS Lett. 201, 267–270
11. Mitchell, C. G. (1996) Biochem. J. 313, 769–774
12. Lindbladth, C., Rault, M., Haggland, C., Small, W. C., Moobach, K., Bulow, L., Evans, C., and Srere, P. A. (1994) Biochemistry 33, 11692–11698
13. Elocq, A. H., and McCammon, J. A. (1996) Biochemistry 35, 12652–12658
14. Srere, P. A., Malloy, C. R., Sherry, A. D., and Sumegi, B. (1995) Adv. Mol. Cell Biol. 11, 125–145
15. Kispal, G., Evans, C. T., Malloy, C., and Srere, P. A. (1989) J. Biol. Chem. 264, 11204–11210
16. Sumegi, B., Sherry, A. D., and Malloy, C. R. (1990) Biochemistry 29, 9106–9110
17. Sumegi, B., Sherry, A. D., Malloy, C. R., and Srere, P. A. (1993) Biochemistry 32, 12725–12729
18. Jones, E. W. (1991) J. Biol. Chem. 266, 7963–7966
19. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
20. Suissa, M., Sadra, K., and Schatz, G. (1984) EMBO J. 3, 1781–1984
21. Cousens, D. J., Wilson, M. J., and Hinchcliffe, E. (1990) Nucleic Acids Res. 18, 1308
22. Hinne, A., Hicks, J. B., and Fink, G. R. (1978) Proc. Natl. Acad. Sci. U. S. A. 75, 1929–1933
23. Brindle, K. M., Williams, S.-P., and Boulton, M. (1989) FEBS Lett. 255, 121–124
24. Srere, P. A., Brazil, H., and Gonen, L. (1963) Acta Chem. Scand. 17, S129–S134
25. Gancedo, J. M., and Gancedo, C. (1973) Biochimie (Paris) 55, 205–211
26. Williams, S.-P., Haggie, P. M., and Brindle, K. M. (1997) Biophys. J. 72, 490–488
27. Rosenkrantz, M., Alam, T., Kim, K.-S., Clark, B. J., Srere, P. A., and Guarente, L. (1986) Mol. Cell. Biol. 6, 4509–4515
28. Stejskal, E. O., and Tanner, J. E. (1965) J. Chem. Phys. 42, 288–292
29. Cantor, C. R., and Shimmel, P. R. (1980) Biophysical Chemistry, W. H. Freeman & Co., San Francisco
30. Freifelder, D. (1982) Physical Biochemistry, W. H. Freeman & Co., San Francisco
31. Velot, C., Mixon, M. B., Teige, M., and Srere, P. A. (1997) Biochemistry 36, 14271–14276
32. McAlister-Henn, L., and Small, W. C. (1992) Prog. Nucleic Acid Res. Mol. Biol. 57, 317–339
33. Jia, Y.-K., Bécam, A. M., and Herbert, C. J. (1997) Mol. Microbiol. 24, 53–59
34. Lewin, A. S., Hines, V., and Small, W. C. (1990) Mol. Cell. Biol. 10, 1399–1405
35. Lindbladh, C., Brodeur, R. D., Lilius, G., Bälow, L., Mosbach, K., and Srere, P. A. (1994) Biochemistry 33, 11684–11691
36. Hubbard, S. J., and Thornton, J. M. (1993) NACCESS, Computer Program, Department of Biochemistry and Molecular Biology, University College, London
37. Sali, A., and Blundell, T. L. (1993) J. Mol. Biol. 234, 779–815
38. Williams, S.-P., Fulton, A. M., and Brindle, K. M. (1993) Biochemistry 32, 4895–4902
39. Aragón, J. J., and Sols, A. (1991) FASEB J. 5, 2945–2950
40. Minton, A. P. (1992) Biophys. J. 63, 1090–1100
41. Fushimi, K., and Verkman, A. S. (1991) J. Cell Biol. 112, 719–725
42. Swaminathan, R., Hoang, C. P., and Verkman, A. S. (1997) Biophys. J. 72, 1900–1907
43. Scalettar, B. A., Abney, J. R., and Hackenbrock, C. R. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 8057–8061
44. López-Beltrán, E. A., Mate, M. J., and Cerdán, S. (1996) J. Biol. Chem. 271, 10648–10653
45. Partikian, A., Olveczky, B., Swaminathan, R., Li, Y., and Verkman, A. S. (1996) J. Cell Biol. 140, 821–829
46. Hutson, S. M., Berkich, D., Williams, G. D., LaNoue, K. F., and Briggs, R. W. (1989) Biochemistry 28, 4325–4332
47. Jeffrey, F. M., Storey, C. J., Nunnelly, R. L., and Malloy, C. R. (1989) Biochemistry 28, 5323–5326
48. Hutson, S. M., Williams, G. D., Berkich, D. A., LaNoue, K. F., and Briggs, R. W. (1992) Biochemistry 31, 1322–1330
49. Masson, S., and Quisterriff, B. (1992) Biochemistry 31, 7488–7493
