Diffusion of Tricarboxylic Acid Cycle Enzymes in the Mitochondrial Matrix in Vivo

EVIDENCE FOR RESTRICTED MOBILITY OF A MULTienzyme COMPLEX*

Peter M. Haggie† and A. S. Verkman
From the Departments of Medicine and Physiology, Cardiovascular Research Institute, University of California, San Francisco, California 94143-0521

It has been proposed that enzymes in many metabolic pathways, including the tricarboxylic acid cycle in the mitochondrial matrix, are physically associated to facilitate substrate channeling and overcome diffusive barriers. We have used fluorescence recovery after photobleaching to measure the diffusional mobilities of chimeras consisting of green fluorescent protein (GFP) fused to the C terminus of four tricarboxylic acid cycle enzymes: malate dehydrogenase, citrate synthase, isocitrate dehydrogenase, and succinyl-CoA synthetase. The GFP–enzyme chimeras were localized selectively in the mitochondrial matrix of transfected Chinese hamster ovary (CHO) and COS7 cells. Laser photobleaching using a 0.7-μm diameter spot demonstrated restricted diffusion of the GFP–enzyme chimeras. Interestingly, all four chimeras had similar diffusional characteristics, ∼45% of each chimera was mobile and had a diffusion coefficient of 4 × 10−8 cm²/s. In contrast, unconjugated GFP in the mitochondrial matrix (targeted using COX8 leader sequence) diffused freely (nearly 100% mobility) with a greater diffusion coefficient of 20 × 10−8 cm²/s. The mobility of the GFP–enzyme chimeras was insensitive to substrate source, ATP depletion, or inhibition of the adenine nucleotide translocase. These results indicate similar mobility characteristics of unrelated tricarboxylic acid cycle enzymes having different sizes and physical properties, providing biophysical evidence for a diffusible multienzyme complex in the mitochondrial matrix.

Mitochondria play a central role in many cellular processes including ATP production, synthesis of metabolic intermediates, apoptosis, and cell signaling (1–4). The tricarboxylic acid cycle is a major biochemical pathway that is localized to the mitochondrial matrix (Fig. 1A). The tricarboxylic acid cycle processes two-carbon units from carbohydrates, amino acids, and fatty acids in the form of acetyl-CoA to generate reducing equivalents (NADH and FADH2) for ATP production by the electron transport chain. Several lines of indirect evidence have suggested that enzymes of the tricarboxylic acid cycle associate to form a functional complex, or metabolon (5). The metabolon has been proposed to facilitate substrate channeling from one enzyme to another, with consequent reduction in metabolite pool sizes, reduction in lag times, increase in metabolic rates, protection of intermediates, and reduction of diffusive barriers (6–9).

In vitro association of matrix enzymes with components of the inner mitochondrial membrane has been demonstrated by a variety of biochemical and molecular approaches (reviewed in Refs. 10–12). Genetic fusions of the sequential tricarboxylic acid cycle enzymes malate dehydrogenase and citrate synthase from yeast (13, 14) and pig (15) gave improved kinetics compared with similar solutions of the unconjugated enzymes. Numerical simulations provided evidence for a continuous surface channel that may facilitate electrostatic channeling of substrate between the active sites of three consecutive tricarboxylic acid cycle enzymes (malate dehydrogenase, citrate synthase, and aconitate) (16, 17). Multiple tricarboxylic acid cycle enzymes have been co-isolated from mammalian cells (18, 19) and lower organisms (20–22). A complex isolated from Saccharomyces cerevisiae by Grandriez-Vazie et al. (22) contained several enzymes from the tricarboxylic acid cycle (malate dehydrogenase, citrate synthase, succinate dehydrogenase, and fumarase) together with enzymes involved in NADH metabolism. Nuclear magnetic resonance studies show preservation of carbon 13 asymmetry in metabolites distal to the symmetrical intermediates succinate and fumarate, supporting the possibility of substrate channeling without aqueous-phase diffusion (23, 24). In yeast, disruption of the genes encoding mitochondrial citrate synthase (CIT1) and malate dehydrogenase give cells that cannot grow on acetate (a purely oxidative energy source) despite the existence of alternative enzymes (such as CIT3) to carry out the same functions (25, 26). Last, the broadening of fluorine 19 resonances from fluorotryptophan-labeled mitochondrial citrate synthase in intact yeast cells has been interpreted in terms of reduced rotational mobility as a consequence of enzyme complexation (27).

There remains, however, controversy about the existence of a tricarboxylic acid cycle enzyme metabolon because of potential dilution and other artifacts during isolations of complexes and the paucity of direct evidence of physical interactions among enzymes in intact cell systems. The purpose of this study was to test for physical associations of tricarboxylic acid cycle enzymes in mammalian cells in vivo by measurement of the diffusional mobilities of GFP-labeled tricarboxylic acid cycle enzyme chimeras. Fluorescence recovery after photobleaching was used to

* This work was supported by Grants EB00415, EY13574, HL60288, HL59198, and DK35124 from the National Institutes of Health and Grant R613 from the Cystic Fibrosis Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
† To whom correspondence should be addressed: 1246 Health Sciences East Tower, Cardiovascular Research Inst., University of California, San Francisco, CA 94143-0521. Tel.: 415-476-8530; Fax: 415-665-3847; E-mail: haggie@itsa.ucsf.edu.

‡ The abbreviations used are: GFP, green fluorescent protein; CS, citrate synthase; MD, malate dehydrogenase; ICD, isocitrate dehydrogenase; SCS, α-succinyl-CoA synthetase; PBS, phosphate-buffered saline; CCCP, carbonyl cyanide m-chlorophenylhydrazine; diOC6(3), 3,3′-dihexyloxycarbocyanine iodide.
measure diffusion. We reasoned that enzymes present in a large, multienzyme complex should have reduced mobility compared with unconjugated GFP and that various unrelated enzymes may have similar diffusional characteristics. We previously applied photobleaching methods to quantify the rheological properties of the cytoplasm and aqueous compartment of intracellular organelles (reviewed in Ref. 28), including the diffusion of unconjugated GFP in the mitochondrial matrix (29). Unexpectedly, it was found that unconjugated GFP diffused freely in the crowded mitochondrial matrix at a rate only 3- to 6-fold higher than in saline. It was proposed that unimpeded GFP diffusion might occur in mitochondrial enzymes bound to the matrix-facing surface of the inner mitochondrial membrane, creating a relatively macromolecule-free aqueous space. We find here that the diffusion of four unrelated tricarboxylic acid cycle enzymes was similar and independent of cell metabolic state, but significantly reduced compared with the diffusion of unconjugated GFP. The results provide in vivo biophysical evidence for physical association among tricarboxylic acid cycle enzymes in living mammalian cells.

EXPERIMENTAL PROCEDURES

GFP-Tricarboxylic Acid Cycle Enzymes—The coding sequences of citrate synthase (CS), malate dehydrogenase (MD), γ-isocitrate dehydrogenase (ICD), and α-succinyl-CoA synthetase (SCS) were PCR-amplified using mouse liver cDNA as template and ligated into mammalian expression plasmid pcDNA3.1 (Invitrogen). CS, ICD, and SCS were cloned at 5'-HindIII and 3' BamHI sites, and MD at 5'-NheI and 3'-HindIII sites. The forward and reverse primers used for PCR amplifications (3'-primers designed to remove stop codons) were (restriction site underlined) as follows: for CS, 5'-CCCGAAGCTTCTAGGGCCTCACTGGACCAACCCGCG and 5'-CCCGGATCCCTCTAGGATTCAGCCTCACTGGACCAACCCGCG; for MD, 5'-CCGGATCCCTCTAGGATTCAGCCTCACTGGACCAACCCGCG and 5'-CCGGATCCCTCTAGGATTCAGCCTCACTGGACCAACCCGCG; for ICD, 5'-CCCAAGCTTCCCTCATGTCCTTGACAAAGTCC; for SCS, 5'-CGGGATCCACAGCCTCCACAGCCCG and 5'-CCCAAGCTTCCCTCATGCTTGACAAAGTCC; for MD, 5'-CCGGATCCCTCTAGGATTCAGCCTCACTGGACCAACCCGCG and 5'-CCGGATCCCTCTAGGATTCAGCCTCACTGGACCAACCCGCG.

Enzyme Assays—Enzyme activities in cell lysates were determined spectrophotometrically using thiobis-(2-nitrobenzoate) (TNB) absorbance (412 nm) for CS and reduced β-nicotinamide adenine dinucleotide (NADH) absorbance (340 nm) for MD. Cells were lysed in PBS containing 1% Triton X-100 (30 min, 4 °C) followed by mechanical disruption (aspiration through a 28-gauge needle) and centrifugation (14,000 x g for 10 min, 4 °C). MD activity was assayed in PBS containing 0.5 mM oxaloacetate and 0.25 mM NADH. CS activity was assayed in PBS containing 0.5 mM oxaloacetate, 0.2 mM acetyl-CoA, and 0.1 mM 5'-dithiothreitol (2-nitrobenzoate). Protein content was measured using the DC protein assay kit (Bio-Rad) for computation of specific activities with bovine serum albumin as standard. Assays were performed on n = 8-9 cultures.

RESULTS

The four enzymes studied here carry out different reactions and are located at different sites in the tricarboxylic acid cycle as shown in Fig. 1A. Fig. 1B shows the tricarboxylic acid enzyme-GFP chimeras used for mammalian cell transfections, along with their putative subunit organizations and oligomeric sizes (without GFP). Unconjugated GFP was targeted to the mitochondrial matrix by fusion downstream from a COX8 leader sequence ((COX8)-GFP), which is partially cleaved after entering the matrix. GFP was fused downstream of MD and CS, which are homodimeric enzymes, and downstream of single subunits of the heteromeric enzymes ICD (γ-subunit) and SCS (α-subunit). Fig. 1C shows a strict mitochondrial pattern of fluorescence in CHO cells after transfection with

vurd Apparatus). Objective broad temperature was maintained at 37 °C using a lens thermoregulator (Biophot). Software written in LabVIEW (National Instruments) was used for data acquisition and analysis.

Spot photobleaching was performed using an inverted epifluorescence microscope with photomultiplier detection. An acousto-optic device was used to modulate the intensity of an argon ion laser beam (488 nm) for 50-70 μs. Coherent bleaching of a brief bleach pulse of 5 μJ/mg increased intensity than the probe beam. The first order beam was directed onto the cells by a dichroic mirror (515 nm) using an oil immersion objective lens (Nikon, 100× or 60×, numerical aperture 1.4). Emitted fluorescence passed through serial filters (530 ± 15 nm band-pass and 515 nm long-pass), detected by a photomultiplier, then converted into a 14-bit analog signal by an analog-to-digital converter (30). Fluorescence was sampled continuously over 200 ms after the bleach pulse, at rates of up to 1 MHz for a specified time after bleaching, and then at 1 Hz (shutter opened for 20 ms per acquisition) for longer times. Fluorescence recovery curves, F(t), were analyzed by non-linear least square fitting using the semi-empirical equation: \( F(t) = F_0 + (F - F_0) \cdot e^{-kt} \), where P is pre-bleach fluorescence, F0 is fluorescence immediately after bleaching, R is the mobile fraction, α is the time exponent (assuming Brownian diffusion α = 1), and t0 is the half-time for recovery (31, 32). Diffusion coefficients (D, in cm²/s) were determined from measured t0 values using a mathematical model to account for mitochondrial geometry (29, 33). Bleach times (generally 10–50 μs) were adjusted to give 20–30% bleach depth. Each recovery curve was generated by averaging recovery curves from 15 to 25 individual cells (no more than one bleach experiment per cell), and each maneuver was performed on four to nine (CHO-K1) or three to five (COX7) different sets of cultures, such that >30 cells were studied for each maneuver. Results are reported as mean ± S.E. for the number (n) of different cultures studied. Statistical analysis was performed by analysis of variance against control conditions (p < 0.05 considered significant).

For photobleaching with image detection, cells were imaged using a Leitz upright fluorescence microscope with Nipkow-wheel confocal module and cooled CCD camera detector (Photometrics). A Nikon 25× long-working-distance air objective focused a shuttered bleach beam (from the argon laser) onto the sample from below, and cells were imaged from above using the oil immersion 60× objective and GFP filter set (HQ filters, Chroma) at prescribed times after the bleach pulse.

Immunoblot Analysis—Immunoblots on mitochondrial lysates from transiently transfected CHO cells, prepared using standard methods (34), were done using a rabbit polyclonal antibodies against GFP (Clontech) and secondary anti-rabbit IgG conjugated to horseradish peroxidase (Amerham Biosciences) for detection by enhanced chemiluminescence (Amerham Biosciences).

Enzyme Assays—Enzyme activities in cell lysates were determined spectrophotometrically using thiobis-(2-nitrobenzoate) (TNB) absorbance (412 nm) for CS and reduced β-nicotinamide adenine dinucleotide (NADH) absorbance (340 nm) for MD. Cells were lysed in PBS containing 1% Triton X-100 (30 min, 4 °C) followed by mechanical disruption (aspiration through a 28-gauge needle) and centrifugation (14,000 x g for 10 min, 4 °C). MD activity was assayed in PBS containing 0.5 mM oxaloacetate and 0.25 mM NADH. CS activity was assayed in PBS containing 0.5 mM oxaloacetate, 0.2 mM acetyl-CoA, and 0.1 mM 5′-dithiothreitol (2-nitrobenzoate). Protein content was measured using the DC protein assay kit (Bio-Rad) for computation of specific activities with bovine serum albumin as standard. Assays were performed on n = 8-9 cultures.

The results show that enzymes present in a large, multienzyme complex should have reduced mobility compared with unconjugated GFP and that various unrelated enzymes may have similar diffusional characteristics.
whether the mitochondrial matrix compartment is contiguous of the cell of a series of images for an MD-GFP-transfected CHO cell. A region that the chimeras remained intact after transfection. Bands at the predicted molecular sizes for the antibody of lysates from CHO cells after transient transfection with each of the five constructs. Similar staining was seen in transfected COS7 cells (not shown). Immunoblot analysis of transfected cells probed with anti-GFP antibody (Fig. 1D) showed predominant bands at the predicted molecular sizes for the respective subunits fused to GFP of (in kDa) —60 (MD-GFP), 75 (CS-GFP), 65 (γ-ICD-GFP), and 60 (α-SCS-GFP), indicating that the chimeras remained intact after transfection. Bands at 27 kDa and slightly larger size were seen for mitochondrial (COX8)-GFP, representing cleaved and non-cleaved moieties. Photobleaching with image detection was done to examine whether the mitochondrial matrix compartment is contiguous in the cell models studied here. Fig. 2A shows a representative series of images for an MD-GFP-transfected CHO cell. A region of the cell of ~5 µm in diameter was bleached (marked by white cross-hairs in the pre-bleach image) by a brief laser pulse, and serial images were collected to visualize the diffusion of unbleached MD-GFP into the darkened zone. Fluorescence recovery (increased fluorescence in bleached region) was not observed for MD-GFP and the other chimeras (and for unconjugated GFP), indicating that the mitochondrial matrix compartment is not contiguous over long distances. Similar results were found in transfected COS7 cells. In contrast, image photobleaching experiments on GFP-labeled endoplasmic reticulum show complete fluorescence recovery (~35), indicating a contiguous luminal compartment.

Spot photobleaching using a much smaller spot size (diameter ~0.7 µm) produced by a 100× objective lens was done to determine the diffusional rate of each enzyme-GFP chimera, as well as the fraction of GFP molecules that were mobile. The smaller spot diameter compared with the length of individual mitochondria (in projection) permitted the observation of fluorescence recovery that could not be seen when a large region was bleached as in Fig. 2A. Fig. 2B shows the fluorescence recovery after spot photobleaching for CHO cells transfected with each of the GFP-labeled enzymes. Data are shown for a 2-s continuous acquisition, followed by a 4–14-s discontinuous acquisition (at 1 data point/s) to identify long term recovery processes. Compared with unconjugated GFP in which fluorescence recovered by >80% with a recovery half-time (t1/2) of ~20 ms (see below, Fig. 3B, left), the fluorescence recovery of the enzyme-GFP chimeras was slowed (t1/2 ~100 ms), and only a fraction of the bleached fluorescence (~40%) showed recovery. Interestingly, the fluorescence recovery curves for the four unrelated enzyme-GFP chimeras were similar in terms of kinetics and extent of recovery. Similar results were found for transfected COS7 cells (Fig. 2C).

Further experiments were done to confirm the diffusive origin of the fluorescence recovery and to measure absolute diffusion coefficients and percentages recovery. As found in previous GFP photobleaching studies (35–37), fluorescence recovery by diffusion-independent processes, such as “reversible photobleaching” involving triplet-state recovery, is generally observed and must be distinguished from diffusion-dependent “irreversible photobleaching.” Photobleaching of paraffin-embedded cells was used to distinguish between diffusion-independent and -dependent fluorescence recovery, since fixation immobilizes GFP. Fig. 3A (four right panels) shows a small, rapid fluorescence recovery (t1/2 ~5 ms) after photobleaching fixed cells transfected with each of the GFP-enzyme chimeras. The insets show data on an expanded time scale. For comparison, fluorescence recovery from unfixed MD-GFP expressing live cells is also shown (left panel). The ~5-ms fluorescence recovery process was confirmed to be diffusion-independent fluorescence recovery by its independence of spot size (0.7–2.0 µm, using 100× and 60× objectives), whereas diffusion-dependent recovery in unfixed cells was strongly spot size-dependent (not shown). Since diffusion-independent recovery was much faster than diffusion-dependent recovery for the enzyme-GFP chimeras under the experimental conditions here, quantitative analysis of diffusion could be done by exclusion of the first 15 ms of the fluorescence recovery.

To determine the absolute diffusion coefficients (D) of the
enzyme-GFP chimeras, comparative measurements were performed for the unconjugated (COX8)-GFP (Fig. 3B, left). The diffusion of unconjugated GFP was substantially faster and more complete than the diffusion of the enzyme chimeras, and similar in CHO and COS7 cells. Values of $D$, computed from $t_{1/2}$ by methods described previously (29, 33) were $(2.0 \pm 0.2) \times 10^{-7} \text{cm}^2/\text{s}$ for CHO cells and $(2.5 \pm 0.3) \times 10^{-7} \text{cm}^2/\text{s}$ for COS7 cells (S.E., $n = 5$). Quantitative determination of percentage fluorescence recovery required a geometric correction for the size of the bleached volume relative to the total volume of the mitochondrial matrix; for example, the apparent percentage recovery can be no greater than 50% if half of the mitochondrial volume is bleached. To determine the geometric correction factor empirically, photobleaching measurements were done after staining the lipidic limiting membranes of mitochondria with the dye diOC$_6$(3) (Fig. 3B, right). We reasoned that the percentage recovery should be 100% if no geometric correction was needed, but <100% when a non-zero fraction of the projected mitochondrial area is bleached. Fig. 3B (right) shows percentages recovery of $86 \pm 3\%$ and $85 \pm 3\%$ ($n = 5–7$) for diOC$_6$(3)-labeled mitochondria in CHO and COS7 cells, respectively. Fig. 3C summarizes diffusion coefficients (top) and corrected percentages recovery (bottom) for unconjugated GFP and for each of the enzyme-GFP chimeras. There was no significant difference in diffusion coefficients or percentages recovery of the four enzyme-GFP chimeras, but these values were much lower than for unconjugated GFP in the mitochondrial matrix.

Control studies were done to investigate whether the fluorescence recovery depended on the amount of expressed enzyme-GFP protein and to estimate the amount and function of the expressed chimera compared with the native enzyme. Fig. 4A (left) shows fluorescence recovery curves of CHO cells expressing different amounts of MD-GFP as indicated by the averaged relative brightnesses. Background-corrected fluorescence intensities varied by 17-fold from brightly to dimly fluorescent cells. Fig. 4A (right) shows no significant effect of the level of MD-GFP expression on deduced percentages recovery or diffusion coefficients. To estimate the function of expressed MD-GFP and CS-GFP relative to endogenous enzyme, functional assays were performed in homogenates of non-transfected versus transfected cells (Fig. 4B). The MD activity of MD-GFP transfected cells was $\sim 25\%$ higher than in non-transfected cells, and CS activity was increased by $\sim 35\%$ in CS-GFP transfected cells. Given a measured transfection efficiency of $\sim 50\%$ in these studies (by counting fluorescent versus total cells using a $60 \times$ oil objective), the average amount of MD and CD overexpression in transfected cells was $\sim 50\%$ and $\sim 70\%$, respectively, of that present endogenously in non-transfected cells. Furthermore, the activity of endogenous CS in MD-GFP transfected cells and of MD in CS-GFP transfected cells was similar to that in non-transfected cells.

To investigate the possibility that the mobility of tricarboxylic acid cycle enzymes is subject to regulation in response to metabolic or other stresses, photobleaching measurements of MD-GFP, CS-GFP, and unconjugated GFP diffusion were made in response to a series of maneuvers. Representative data for MD-GFP and CS-GFP are shown in Fig. 5, A and B, respectively, and averaged diffusion coefficients and percentages mobility for all three GFPs are summarized in Table I. Substrate was varied from a glycolytic source (6 mM glucose, as in Figs. 2 and 3) to an oxidative source (6 mM pyruvate, first curves), as well as a mixed glycolytic/oxidative source (6 mM glucose + 1 mM pyruvate, second curves). There was no significant effect of substrate on diffusion coefficient or percentage recovery. Similar results were obtained for the diffusion of ICD and SCS (not shown). The inhibitors of metabolism 2-deoxyglucose (without other substrate, third curves) and iodoacetic acid (fourth curves) also had no significant effect on the diffusion of MD-GFP, CS-GFP, or unconjugated GFP in the mitochondrial matrix. Inhibition of the adenine nucleotide translocase with atractysode also did not affect enzyme diffusional mobility (fifth curves). Collapse of the mitochondrial membrane potential with the protontophore CCCP, or increasing mitochondrial calcium concentration with the ionophore A23187, resulted in
rounding of mitochondria (Fig. 5C, right) and apparent immobilization of the enzyme-GFP chimeras and unconjugated GFP (example shown in Fig. 5C, left). Similarly, inhibition of the mitochondrial ATPase by oligomycin resulted in extensive mitochondrial fragmentation and rounding. Meaningful evaluation of GFP diffusion is not possible after mitochondrial calcium elevation or collapse of the pH/potential gradient, because the fragmented/rounded mitochondria are comparable in size or smaller than the laser spot diameter.

**DISCUSSION**

As described in the Introduction, a substantial body of indirect evidence suggests that enzymes in the tricarboxylic acid cycle assemble in multienzyme complexes to facilitate metabolism. The consequent channeling of substrate from active site to active site, by direct transfer or concentrating effects, is proposed to reduce metabolite pool sizes, reduce lag times, increase metabolic rates, protect labile intermediates, and overcome diffusive barriers (6–9). However, the evidence for a
multienzyme complex has a number of potential weaknesses. For example, isolation of enzyme complexes (as described in Refs. 21 and 22) depends on the details of isolation conditions and may be affected by the greatly diluted state after mitochondrial disruption. The preservation of carbon 13 asymmetry despite conversion through symmetrical intermediates (23, 24) could be interpreted in terms of transient complexation of a tricarboxylic acid cycle enzyme with the previous enzyme-substrate complex. The restricted rotational mobility of an enzyme (27) can be interpreted in terms of enzyme binding to the mitochondrial inner membrane rather than complexation with other enzymes. The increased metabolic rate after genetic fusion of successive enzymes in a pathway (13–15) is not surprising and does not prove that complexation occurs in vivo. Finally, the rapid diffusion of unconjugated GFP in the mitochondrial matrix (29) suggests that metabolite channeling may not be required to overcome diffusive barriers if metabolites, like the much larger GFP, are able to diffuse rapidly in the matrix. Thus, although enzyme complexation and metabolite channeling is an attractive hypothesis, the evidence for the existence of complexes in vivo is not conclusive.

We used photobleaching recovery to measure the diffusional mobilities of four tricarboxylic acid cycle enzymes. Two of the enzymes (MD and CS) assemble as homodimers and the other two (ICD and SCS) as heterotetramers. These enzymes were chosen as having different sizes and structures, as well as different sites in the tricarboxylic acid cycle (see Fig. 1A). In addition, a practical consideration was that the C terminus GFP fusions for these enzyme subunits did not affect mitochondrial targeting or function; indeed, GFP fusions were unsuccessful for several other subunits of tricarboxylic acid cycle enzymes. Transfection of mammalian cells with cDNAs encoding each of the four enzyme-GFP chimeras yielded mitochondria-specific staining. Control studies showed that enzyme-GFP diffusion was not dependent on the level of expression and that the amount of added enzyme by transfection was relatively small compared with the amount of endogenous enzyme.

The photobleaching experiments indicated that each of the four tricarboxylic acid cycle enzymes was present in two pools, an apparently immobile pool that did not diffuse measurably (in a 0.7 μm spot over at least 14 s) and a mobile pool with diffusion coefficient ~4-fold lower than that of unconjugated GFP. Given the dependence of diffusion coefficient for a spherical molecule on the cube root of molecular size, 4-fold slowed diffusion (compared with unconjugated GFP, 27 kDa) indicates that the putative complex may have a molecular size as large as ~1.7 MDa (4^3 × 27 kDa), which is comparable with the total molecular size of all tricarboxylic acid cycle enzymes together. The most interesting observation here was that in two different cell lines the diffusive mobilities of the four tricarboxylic acid cycle enzymes were not significantly different from one another. The simplest interpretation of the similar mobilities of the unrelated enzymes, and their much slower mobilities compared with unconjugated GFP, is that the enzymes exist as components of a multienzyme complex. The multienzyme complex appeared to be immobilized ~50% of the time, possibly by binding to the inner mitochondrial membrane. It is possible that succinate dehydrogenase, the only integral membrane protein in the tricarboxylic acid cycle, is a site of immobilization of a fraction of the putative multienzyme complex. However, the relatively rapid diffusion of the mobile component, compared with that predicted for a membrane protein, suggests that the putative complex cannot be permanently complexed to succinate dehydrogenase. In a previous study, we found that two components of the mitochondrial fatty acid β-oxidation multienzyme complex in the matrix were essentially immobile (29), in contrast to the tricarboxylic acid cycle enzymes studied here. We speculate that immobilization of enzymes, either partial or complete, is a com-

![Figure 5](https://example.com/figure5.png)

**Figure 5.** Effect of putative modulators of metabolism and mitochondrial function on the diffusion of MD-GFP (A) and CS-GFP (B) in CHO cells. Representative photobleaching recovery curves shown for bleach conditions as in Fig. 2B. Maneuvers (concentrations, incubation times) are described under “Experimental Procedures.” See Table I for summary of fitted diffusion coefficients and percentages recovery. C, recovery curve for MD-GFP-expressing cells after incubation with A23187 (left), with fluorescence micrographs for indicated maneuvers.

**Table I**

| Modality | Malate dehydrogenase | Citrate synthase | (COX8)-GFP | Malate dehydrogenase | Citrate synthase | (COX8)-GFP |
|----------|----------------------|------------------|------------|----------------------|------------------|------------|
|          | %                    |                  |            | cm^2/s × 10^-4       |                  |            |
| Glucose 6 mM | 46 ± 2               | 44 ± 1           | 94 ± 3     | 4.0 ± 0.6            | 3.3 ± 0.3       | 20.0 ± 0.3 |
| Glucose 6 mM + pyruvate 1 mM | 44 ± 2               | 44 ± 2           | 92 ± 1     | 4.1 ± 0.5            | 3.5 ± 0.1       | 22.4 ± 0.3 |
| Pyruvate 6 mM | 44 ± 2               | 39 ± 2           | 91 ± 2     | 4.2 ± 0.8            | 3.6 ± 0.5       | 21.4 ± 0.3 |
| 2-Deoxyglucose 6 mM | 42 ± 3               | 45 ± 4           | 93 ± 3     | 3.8 ± 0.2            | 3.8 ± 0.6       | 20.2 ± 0.2 |
| Idoxalic acid 100 μM | 46 ± 2               | 43 ± 3           | 91 ± 2     | 5.1 ± 0.5            | 4.7 ± 0.6       | 17.3 ± 0.3 |
| Atractyloside 5 μM | 44 ± 2               | 39 ± 1           | 91 ± 2     | 4.2 ± 0.6            | 3.8 ± 0.4       | 22.5 ± 0.3 |

Data are the mean ± S.E. (n = 4–9 sets of measurements), each measurement the average of 8–15 recovery curves from different cells) for indicated maneuvers (see “Experimental Procedures”). Differences are not significant (compared with glucose control).
mon feature of mitochondrial enzymes. However, the biophysical data here provide no information about the functional significance of partial enzyme immobilization or of the putative enzyme complex.

Based on observations suggesting that the mobilities of glycolytic enzymes in cytoplasm are subject to regulation by metabolic state (38), the hypothesis was tested that metabolic or other stresses might alter the mobility of tricarboxylic acid cycle enzymes. We speculated that regulated mobility could provide a novel mechanism by which metabolic rates might be controlled. However, significant differences were not found in a series of maneuvers for the diffusion coefficients or percentages of un conjugated GFP or the enzyme-GFP chimeras. Changes in glucose/pyruvate concentrations were used to alter metabolic state by providing oxidative versus glycolytic substrates (39). Inhibition of cellular metabolism/ATP depletion was accomplished by 2-deoxyglucose and iodoacetic acid (37, 39, 40), and inhibition of the adenine nucleotide translocase was accomplished by atractyloside. The insensitivity of enzyme-GFP diffusion to these maneuvers suggests that the putative tricarboxylic acid cycle enzyme complex is relatively stable. More severe mitochondrial stress (protonophore CCCP, Ca\(^{2+}\) ionophore A23187, oligomycin) produced major structural changes and apparent enzyme immobilization, although fragmentation of mitochondria precluded meaningful determina tion of GFP mobilities.

In summary, this study provides biophysical evidence of complexation and immobilization of enzymes in the tricarboxylic acid cycle in eukaryotic cells in vivo. However, the nature of the complex cannot be addressed by the diffusion measurements here, nor can its functional significance. The enzyme-GFP expression system and the demonstration of partial mobility establishes an experimental basis to utilize two-color fluorescence cross-correlation spectroscopy (41, 42) for definitive biophysical demonstration of co-diffusion and in vivo complexation of tricarboxylic acid cycle enzymes.

REFERENCES
1. Green, D. R., and Reed, J. C. (1998) Science 281, 1309–1312
2. Duchen, M. R. (2000) J. Physiol. (Lond.) 529, 57–68
3. Saraste, M. (1999) Science 283, 1488–1493
4. Rizzuto, R. (2001) Curr. Opin. Neurobiol. 11, 306–311
5. Srere, P. A. (1985) Trends Biochem. Sci. 10, 109–110
6. Welch, G. R., and Easterby, J. S. (1994) Trends Biochem. Sci. 19, 193–197
7. Anderson, R. S. (1999) Methods Enzymol. 308, 111–145
8. Hochachka, P. W. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 12233–12239
9. Spirey, H. O., and Ovadi, J. (1999) Methods 19, 306–321
10. Srere, P. A. (1987) Annu. Rev. Biochem. 56, 89–124
11. Westerhoff, H. V., and Welch, G. R. (1992) Curr. Top. Cell Regul. 33, 361–390
12. Srere, P. A., Sherry, A. D., Malloy, C. R., and Sumegi, B. (1997) Channeling and Intermediary Metabolism (Aquis, L., and Sherratt, H. S., eds) pp. 201–217, Portland Press Ltd., London
13. Lindblad, C., Bredero, R. D., Lilliu, G., Bulow, L., Mosbach, K., and Srere, P. A. (1994) Biochemistry 33, 11684–11691
14. Lindblad, C., Rauti, M., Haggland, C., Small, W. C., Mosbach, K., Bulow, L., Evans, C., and Srere, P. A. (1994) Biochemistry 33, 11692–11698
15. Shatalin, K., Lebreton, S., Rault-Leonardon, M., Volot, C., and Srere, P. A. (1999) Biochemistry 38, 881–889
16. Elcock, A. H., and McCammon, J. A. (1996) Biochemistry 35, 12652–12658
17. Velot, C., Mixen, M. B., Tiege, M., and Srere, P. A. (1997) Biochemistry 36, 14271–14276
18. Robinson, J. B., and Srere, P. A. (1985) J. Biol. Chem. 260, 10800–10805
19. Robinson, J. B., Inman, L., Sumegi, B., and Srere, P. A. (1997) J. Biol. Chem. 262, 1766–1790
20. Barnes, S. J., and Weitzman, P. D. J. (1986) FEBS Lett. 201, 267–270
21. Mitchell, C. G. (1996) Biochem. J. 313, 769–774
22. Grandvaux-Vaneille, X, Bathan, K., Chaingepain, S., Camougrand, N., Manon, S., and Schmitter, J. M. (2001) Biochemistry 40, 9758–9769
23. Sumegi, B., Sherry, A. D., and Malloy, C. R. (1992) Biochemistry 31, 9106–9110
24. Sumegi, B., Sherry, A. D., Malloy, C. R., and Srere, P. A. (1993) Biochemistry 32, 12725–12729
25. Kaspal, G., Evans, C. T., Malloy, C., and Srere, P. A. (1989) J. Biol. Chem. 264, 11294–112910
26. Velot, C., Lebreton, S., Murgunov, I., Usher, K. C., and Srere, P. A. (1999) Biochemistry 38, 16195–16204
27. Haggie, P. M., and Brindle, K. M. (1999) J. Biol. Chem. 274, 3941–3945
28. Verkman, A. S. (2002) Trends Biochem. Sci. 27, 27–33
29. Partikian, A., Olevczky, B., Swaminathan, R., Li., Y., and Verkman, A. S. (1998) J. Cell Biol. 149, 821–829
30. Verkman, A. S., Vetrivel, L., and Haggie, P. M. (2001) Methods in Cellular Imaging (Periasamy, A., ed) pp. 112–127, Oxford University Press, Oxford
31. Feder, T. J., Brust-Mascher, I., Slattery, J. P., Baird, B., and Webb, W. W. (1996) Biophys. J. 70, 2367–2373
32. Periasamy, N., and Verkman, A. S. (1996) Biophys. J. 75, 557–567
33. Olevczky, B. P., and Verkman, A. S. (1998) Biophys. J. 74, 2722–2730
34. Kawasaki, K., Kuge, O., Yamakawa, Y., and Nishijima, M. (2001) Biochem. J. 354, 9–15
35. Dayer, M. J., Hom, E. F., and Verkman, A. S. (1999) Biophys. J. 76, 2843–2861
36. Swaminathan, R., Hoang, C. P., and Verkman, A. S. (1997) Biophys. J. 72, 1900–1907
37. Haggie, P. M., Stanton, B. A., and Verkman, A. S. (2002) J. Biol. Chem. 277, 16419–16425
38. Pagliaro, L., and Taylor, D. L. (1992) J. Cell Biol. 118, 859–863
39. Jouaville, L. S., Pinton, P., Bastianutto, C., Rutter, G. A., and Rizzuto, R. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 13807–13812
40. Wu, Y., Sunn, F. F., Tong, D. M., and Taylor, B. M. (1996) Biophys. J. 71, 91–100
41. Hom, E. F., and Verkman, A. S. (2002) Biophys. J. 83, 533–546
42. Hess, S. T., Huang, S., Heikal, A. A., and Webb, W. W. (2002) Biochemistry 41, 697–705