

# Dynamics, structure, and function are coupled in the mitochondrial matrix

BETHE A. SCALETTAR\*, JAMES R. ABNEY†, AND CHARLES R. HACKENBROCK

Laboratories for Cell Biology, Department of Cell Biology and Anatomy, University of North Carolina, Chapel Hill, NC 27599-7090

Communicated by Mary Ellen Jones, June 17, 1991 (received for review March 26, 1991)

**ABSTRACT** The coupling between molecular diffusion and the structure and function of the rat liver mitochondrial matrix was explored using fluorescence anisotropy techniques and electron microscopy. The results confirm that matrix ultrastructure and the concentration of matrix protein are influenced by the respiratory state of mitochondria and the osmolarity of the external medium. At physiological osmolarity, a fluorescent metabolite-sized probe was found to diffuse slowly in the mitochondrial matrix but not to be completely immobile. In addition, significant differences in diffusion rates were found to exist between different mitochondrial respiratory states, with the slowest diffusion occurring in states with the highest matrix protein concentration. These data support the concept of a matrix structure in which diffusion is considerably hindered due to limited probe-accessible water and further suggest that volume-dependent regulation of matrix protein packing may modulate metabolite diffusion and, in turn, mitochondrial metabolism.

In recent years it has become apparent that biological systems contain high concentrations of macromolecules and thus differ markedly from the dilute *in vitro* solutions typically used in experimental studies (1). No biological system better exemplifies this point than the mitochondrial matrix, which is the site of many important oxidative reactions, including those involved in the tricarboxylic acid cycle and fatty acid oxidation pathway. It has been suggested that the concentration of protein in the matrix is so high that it may approach the close-packing limit, thereby creating an unusual "aqueous" milieu in which the motion of metabolic substrates and protein is unusually hindered (2, 3). Moreover, it has been further speculated that such hindered motion would require matrix enzymes to be structurally organized and metabolites to be "channeled" between adjacent enzymes, a process that might accelerate the rates of enzymatic reactions (3, 4). Despite these intriguing possibilities, relatively little is known about the mitochondrial matrix, and elucidating the relationship between metabolism and matrix properties thus remains a key problem in biochemistry.

It is known from electron microscopy studies that mitochondria can exist in two distinct ultrastructural configurations, frequently termed condensed and orthodox (Fig. 1) (5, 6). In rat liver, the matrix of the condensed mitochondrion occupies about 50% of the total mitochondrial volume and contains about 56% (wt/vol) protein, whereas the matrix of the orthodox mitochondrion occupies nearly the entire mitochondrial volume and contains about 26% protein (7). These mitochondrial configurations are dependent on the osmolarity of the external medium and on respiratory state—i.e., whether or not the mitochondria are active in electron transport and ATP synthesis (5, 6, 8).

Unfortunately, it has been difficult to proceed beyond the level of ultrastructure to study matrix properties at the

molecular level. One of the major impediments to such research has been the fact that the matrix is surrounded by the outer and inner mitochondrial membranes, the latter of which is highly impermeable. Thus, it has been difficult to introduce chemical probes into the matrix of intact functional mitochondria, and generally one has been left with the alternative of disrupting the mitochondria partially or completely to study matrix properties. Studies of disrupted matrix preparations have proven useful; they have, for example, suggested that some matrix enzymes interact specifically with one another and with proteins of the inner mitochondrial membrane (for review, see ref. 4). However, in such studies the proteins are removed from their native environment, and thus it is difficult to determine the effects that high matrix protein concentrations have on matrix properties.

We have overcome the need for disruptive procedures by exploiting newly developed methods that permit fluorescent molecules to be introduced into the matrix in a nonperturbing manner [refs. 9–11 (and references cited therein)]. Once fluorescent molecules have been introduced into the matrix, any of a number of different fluorescence techniques can be used to extract interesting information about matrix properties. For example, by monitoring fluorescence spectra, the pH of the matrix can be followed as a function of mitochondrial respiratory state (10). Alternatively, we show that steady-state fluorescence anisotropy measurements can be used to probe diffusion and viscosity in the matrix as a function of mitochondrial configuration or, equivalently, matrix protein concentration. We assume that the diffusion of our fluorescent probe will approximately mirror the diffusion of similarly sized metabolic substrates because, in an environment in which free space is limited, size will largely dictate diffusive rates (12). This work thus represents an attempt to measure an effective viscosity for the matrix and thereby to identify the effects that high matrix protein concentrations have on matrix structure and function.

## THEORY

Steady-state fluorescence anisotropy measurements provide a standard way to determine the effects that environment has on molecular dynamics (13–15). Specifically, once the steady-state anisotropy  $r$  is known, the rotational correlation time of a molecule  $\tau_R$  can be calculated from the relationship (16)

$$r = r_0 / (1 + \tau_F / \tau_R), \quad [1]$$

where  $\tau_F$  is the fluorescence lifetime of the fluorophore and  $r_0$  is the limiting anisotropy of the completely immobilized fluorophore. Rotational diffusion measurements are useful

Abbreviation: mOsm, milliosmolar.

\*Present address: Department of Biochemistry and Biophysics, University of California, San Francisco, CA 94143-0554.

†Present address: Cardiovascular Research Institute, University of California, San Francisco, CA 94143-0532.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

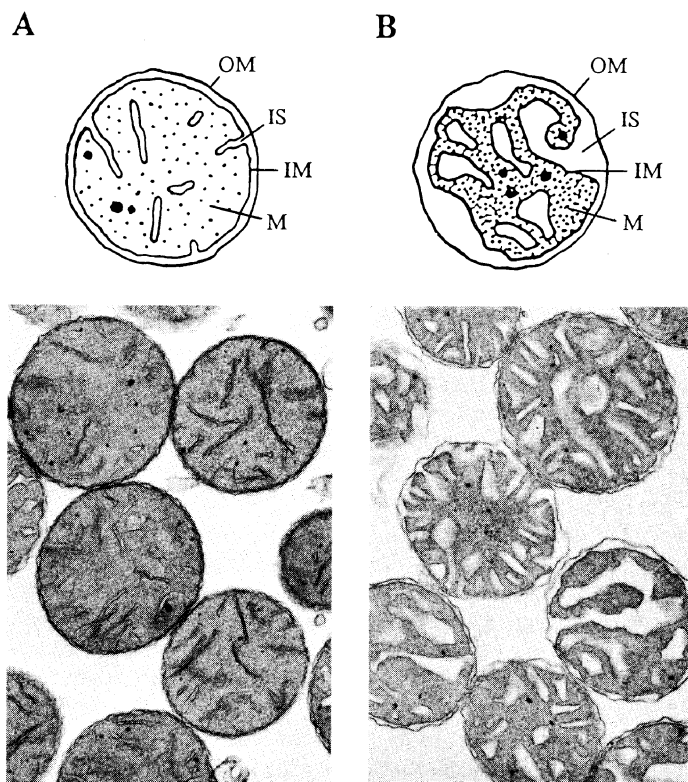


FIG. 1. Electron micrographs and schematic drawings of fluorescently labeled orthodox and condensed (5, 6) rat liver mitochondria. Mitochondria were loaded with fluorophore and then resuspended in Hepes-buffered medium with an osmolarity of 120 or 300 mOsm. Mitochondria were then fixed in suspension with glutaraldehyde [120 mOsm sample, 1.2% (vol/vol) glutaraldehyde in 40 mM sodium phosphate (pH 7.4); 300 mOsm sample, 2% glutaraldehyde in 100 mM sodium phosphate (pH 7.4)], centrifuged into micropellets, and post-fixed with  $\text{OsO}_4$  [2% (wt/vol) in the same phosphate buffers used for glutaraldehyde fixation]. Micropellets were embedded in Epon, and sections were stained with uranyl acetate and lead citrate. (A) Mitochondria from the sample at 120 mOsm were in the orthodox configuration: the matrix volume (M) is large (matrix protein concentration, 260 mg/ml) and the intermembrane space (IS) is small. Also indicated are the outer membrane (OM) and inner membrane (IM). (B) Mitochondria from the sample at 300 mOsm were in the condensed configuration: the matrix is decreased in size (matrix protein concentration, 560 mg/ml) and there is a large intermembrane space. Mitochondrial configuration is also dependent on respiratory state (5, 6). In 300 mOsm medium, state IV (respiratory substrate and  $\text{O}_2$ ) mitochondria are orthodox, state III (respiratory substrate and  $\text{O}_2$  and ADP) mitochondria are fairly condensed, and state IV(i) (respiratory substrate and  $\text{O}_2$  and respiratory inhibitor) mitochondria are very condensed (see text for the functional definitions of these states). We have confirmed that these same ultrastructural configurations occur in respiring fluorescently labeled mitochondria (electron micrographs not shown). ( $\times 19,500$ )

because  $\tau_R$  is proportional to an effective solution "viscosity,"  $\eta$  (15). Thus, as protein concentration and hence viscosity increase, rotational diffusion slows and the anisotropy increases.

## MATERIALS AND METHODS

**Isolation and Fluorescence Loading of Mitochondria.** Mitochondria from the livers of male Sprague-Dawley rats were isolated essentially as described in ref. 17. The mitochondria were loaded with fluorescent probe within 6 hr of harvesting by incubating mitochondria at a protein concentration of 25 mg/ml in 300 milliosmolar (mOsm) medium (see below) with 50  $\mu\text{M}$  carboxyfluorescein diacetate acetoxymethyl ester for 10 min at 15°C. (It was demonstrated that data were independent of loading level.) Carboxyfluorescein diacetate acetoxymethyl ester is nonfluorescent and membrane permeant

until its acetate and acetoxymethyl ester groups are cleaved by nonspecific esterases in the mitochondrial matrix. It is then converted into the intensely fluorescent compound carboxyfluorescein, which is charged and membrane impermeant. Uncleaved carboxyfluorescein diacetate acetoxymethyl ester was removed by centrifuging the mitochondria at  $10,000 \times g$  for 15 min at 4°C and removing the supernatant. Mitochondria were always inspected after labeling using a combined phase-contrast/fluorescence microscope to evaluate their appearance and to verify that the fluorescence was trapped in the mitochondria (Fig. 2).

**Activity Assay.** Mitochondrial function was assayed by determining the respiratory control ratio (i.e., the ratio of the rate of oxygen consumption by mitochondria in the presence and absence of ADP) (18). Measured respiratory control ratios of labeled mitochondria used in these studies were essentially identical to those of control unlabeled mitochondria; typically, ratio values were  $\approx 6$  when succinate was used as the respiratory substrate, indicating that both labeled and unlabeled mitochondria retained substantial function.

**Fluorescence Spectroscopy.** Steady-state anisotropy measurements were made at 10°C on a Perkin-Elmer model 650-40 spectrofluorimeter equipped with the requisite polarization accessories. In the studies described below, labeled mitochondria were suspended at a protein concentration of  $\leq 0.25$  mg/ml in the appropriate buffer. Fluorescence from these samples was excited with light at a wavelength of 490 nm; emission was monitored at 520 nm. Proper polarizer alignment was routinely verified by measuring the anisotropy (ideally one) from a dilute glycogen solution. The appropriate standard (G factor) corrections for nonidealities in the behavior of the detection system were made (15).

The anisotropy was determined from the relationship (14, 15)

$$r = (I_{\parallel}^f - I_{\perp}^f) / (I_{\parallel}^f + 2I_{\perp}^f), \quad [2]$$

where  $I_{\parallel}^f$  and  $I_{\perp}^f$  are the fluorescence intensities obtained when the polarizers on the excitation and emission are parallel and perpendicular, respectively. Since mitochondria scatter light, the total (t) signal from labeled samples is the sum of scattering (s) and fluorescence (f). Scattering was measured by preparing nonfluorescent but otherwise identical "blank" samples, and  $I_{\parallel}^f$  and  $I_{\perp}^f$  were computed using  $I_{\parallel}^f = I_{\parallel}^t - I_{\parallel}^s$  and  $I_{\perp}^f = I_{\perp}^t - I_{\perp}^s$  (16). Under our experimental conditions, the scattering/background correction changed the anisotropy by 0.02 or less.

The limiting anisotropy and fluorescence lifetime of the probe were measured as follows. The limiting anisotropy of

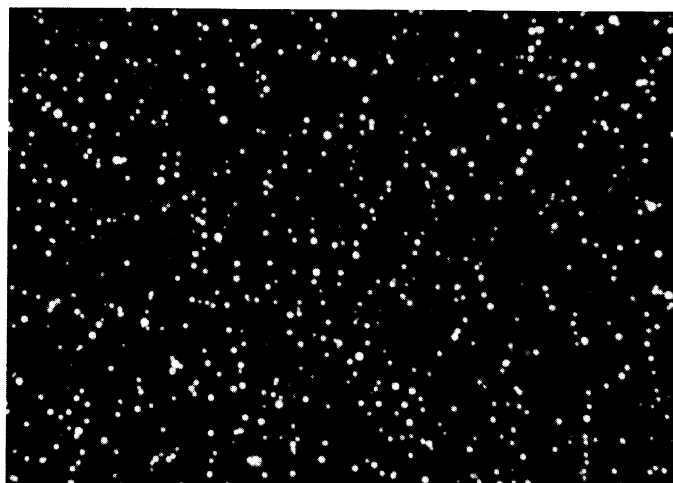


FIG. 2. Fluorescently labeled rat liver mitochondria. The labeled mitochondria shown here were resuspended in 300 mOsm medium. ( $\times 540$ .)

the immobilized fluorophore  $r_0$  was measured by placing carboxyfluorescein in a solution of very high viscosity [98% (vol/vol) glycerol] and then carrying out the anisotropy measurements at low temperature to increase further the viscosity of the solution. The value for  $r_0$  of 0.39 obtained here is close to the theoretical maximum 0.40. Phase-modulation lifetime measurements made on an SLM 48000 spectrofluorimeter (see ref. 15, pp. 51–91) showed that carboxyfluorescein in the matrix of intact mitochondria had a single fluorescence lifetime,  $\tau_F$ , of 3.5 nsec.

To compute normalized effective viscosities, rotational correlation times  $\tau_R$  were calculated from the measured anisotropies  $r$  using the relationship  $\tau_R = \tau_F / (r_0/r - 1)$ , which is just a recast form of Eq. 1. The rotational correlation times thus computed were then normalized to the rotational correlation time of carboxyfluorescein in dilute nonviscous buffer,  $\tau_0 = 200$  psec. Since viscosity and rotational correlation time are directly proportional, the normalized rotational correlation time  $\tau_R/\tau_0$  is equal to the normalized effective viscosity  $\eta/\eta_0$ , where  $\eta_0$  is the viscosity of dilute nonviscous buffer.

**Media for Osmolarity Studies.** Hepes-buffered media (pH 7.4) were prepared at various osmolarities. The 400 mOsm medium contained 2 mM Hepes, 296 mM mannitol, 94 mM sucrose, and defatted bovine serum albumin (0.5 mg/ml). The 300 mOsm medium contained 2 mM Hepes, 220 mM mannitol, 70 mM sucrose, and defatted bovine serum albumin (0.5 mg/ml). Media of osmolarity between 300 and 120 were obtained by appropriately diluting the 300 mOsm medium with 2 mM Hepes buffer. Lower osmolarity media were prepared independently and contained 2 mM Hepes, as well as mannitol and sucrose in the same molar ratio as the 300 mOsm medium.

**Media for Respiratory-State Studies.** The data on respiratory states were obtained by suspending labeled mitochondria at a protein concentration of  $<0.5$  mg/ml in the 300 mOsm medium described above and adding 5 mM  $P_i$ , 2.5 mM  $MgCl_2$ , and 5 mM succinate (the oxidation substrate). This was the state IV medium. The state IV(i) medium was obtained by adding the electron transport inhibitor antimycin A (0.5  $\mu$ g/ml) to the state IV medium; the state III medium

was obtained by adding the phosphorylation substrate ADP (2.5 mM) to the state IV medium. The mitochondria were allowed to respire by placing them at room temperature and stirring for 20 min to ensure that the sample did not become anaerobic as the mitochondria consumed oxygen. The pH of the external medium did not change during the respiratory-state studies.

## RESULTS

We have found that esterases in the matrices of isolated rat liver mitochondria will rapidly cleave the molecule carboxyfluorescein diacetate acetoxyethyl ester and thereby produce the membrane impermeant fluorophore carboxyfluorescein, which is well retained by the matrix for at least 1 day (Fig. 2). Moreover, we have found that carboxyfluorescein loading does not significantly perturb the function of mitochondria or their ability to undergo configurational changes (Fig. 1). We were thus able to use carboxyfluorescein fluorescence to probe the environment of small molecules in the matrix of structurally and functionally intact rat liver mitochondria.

**Osmolarity Studies.** We have monitored ultrastructure and carboxyfluorescein rotational diffusion in the mitochondrial matrix as a function of external osmolarity (Fig. 3). At the highest osmolarities (300–400 mOsm), the mitochondria were in the condensed configuration and the anisotropy was large. This result indicates that rotational diffusion is significantly hindered in the protein dense matrix of the condensed configuration. As the osmolarity was lowered the mitochondria became more orthodox in appearance and the anisotropy fell measurably, indicating that diffusion became progressively more rapid as the matrix protein concentration decreased. Below about 120 mOsm, structural alterations occurred in which the outer membrane was ruptured and the matrix compartment was highly swollen. At this point the anisotropy began to fall markedly. When the osmolarity was decreased to about 40 mOsm or less, the inner membrane unfolded completely into a spherical configuration (19), the matrix density decreased further, and between 25 mOsm and 2 mOsm the value of the anisotropy approached zero.

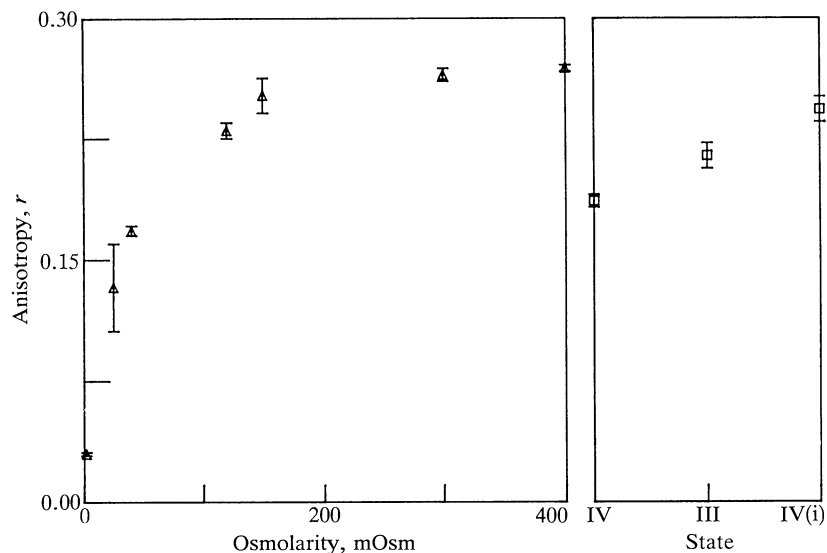


FIG. 3. Anisotropies vs. osmolarity and respiratory state. Error bars reflect the variation observed in data obtained from liver mitochondria harvested from various rats on different days. The effect that medium osmolarity (*Left*) and respiratory state (*Right*) have on carboxyfluorescein rotational diffusion in the matrix is shown. Note that, despite the fact that state IV mitochondria and mitochondria in 120 mOsm medium have a similar (orthodox) ultrastructural configuration, their anisotropies are different. This tends to indicate that there may be subtle structural differences under these two conditions that are not manifest at the level of ultrastructure but that are manifest in the diffusion of carboxyfluorescein.

**Respiratory-State Studies.** We have also monitored ultrastructure and carboxyfluorescein rotational diffusion in the mitochondrial matrix as a function of respiratory state at physiological (300 mOsm) osmolarity (Fig. 3). Electron microscopy and fluorescence anisotropy measurements showed that during electron transport driven by the oxidation of an appropriate respiratory substrate such as succinate (state IV; ref. 20), the mitochondria became orthodox, and carboxyfluorescein diffusion in the matrix compartment was moderately hindered. In contrast, when ATP synthesis was initiated by adding ADP (state III), the mitochondria adopted a more condensed configuration, matrix density increased, and carboxyfluorescein diffusion was correspondingly more hindered. Finally, when electron transport was inhibited by adding antimycin A [state IV(i)], the mitochondria adopted a highly condensed configuration, and carboxyfluorescein diffusion was hindered further.

**Control Experiments for Dye Binding.** Control experiments were conducted that demonstrated that the anisotropy probably did not reflect fluorophore binding to the inner membrane or matrix protein. Specifically, we added carboxyfluorescein to samples containing isolated matrix protein or inside-out inner membrane vesicles (isolations followed the procedures outlined in ref. 21). For both control samples, the measured anisotropy was quite low (0.04), a result that indicates that high anisotropies in the intact matrix (0.23–0.27) are not due primarily to dye binding. In addition, carboxyfluorescein has a single fluorescence lifetime in the matrix, a fact that is suggestive of a single fluorophore population, rather than both bound and unbound populations.

**Control Experiments for Dye Leakage.** Two types of control experiments were also conducted that demonstrated that dye leakage from the mitochondria did not significantly affect the anisotropy. (Dye leakage will lower the measured anisotropy since escaped dye will rotate without hindrance.) (i) We tested for reversibility of anisotropy loss in samples exposed to osmolarities below 300 mOsm by raising the osmolarity back to 300 mOsm. As long as the lowest osmolarity experienced was  $\geq 40$  mOsm, anisotropy loss was totally reversible; below about 40 mOsm it was not. The reversibility test suggests that experimental decreases in anisotropy are not due to dye leakage when the lowest osmolarity experienced is greater than about 40 mOsm, since simply raising the osmolarity should not cause leaked dye to return to the matrix. (ii) We have also centrifuged the mitochondria and

compared the amount of fluorescence in the supernatant and pellet. This assay is, however, subject to some uncertainty in interpretation because 100% of the mitochondria do not always sediment, and disruption of some mitochondria always occurs during resuspension and centrifugation. Nevertheless, the results again show that fluorescence leakage is negligible at or above 120 mOsm and  $\leq 20\%$  down to 40 mOsm. To account for the observed anisotropy change at 40 mOsm purely in terms of dye leakage, about 50% of the dye would have to leave the matrix.

## DISCUSSION

The data presented here can be used to determine the effects that high matrix protein concentrations have on molecular dynamics and structure in the matrix. These effects, in turn, give insight into matrix function.

**Implications for Molecular Dynamics in the Matrix.** A key question surrounding the matrix has been how protein concentration influences rates of rotation and translation of metabolites and enzymes in the matrix (3). This issue has generated interest because rates of enzyme–substrate interactions are influenced by the time it takes these molecules to orient and collide. Therefore, we have calculated the rate of rotation of carboxyfluorescein in the matrix from our anisotropy data by using Eq. 1. These results were then used to calculate effective viscosities for the matrix (ref. 22 and references cited therein; Fig. 4). We find that, in the osmotically induced condensed and orthodox mitochondrial configurations, the effective viscosities of the matrix are 37 and 25 times larger than the viscosity of dilute buffer, respectively. This is quite a striking result; it shows that the mitochondrial matrix is much more viscous than cytoplasm, where viscosities are found to be 3–13 times that of dilute buffer (22). Our viscosity results suggest that the time scale associated with the orientation of a substrate with respect to an enzyme will be lengthened considerably in the intact matrix. Moreover, although our results rigorously apply only to rotation, it is usually observed that translation is more hindered in concentrated solutions than is rotation (12). Thus, the data suggest that translation and collisional interactions in the mitochondrial matrix will also be significantly impeded. In fact, since some metabolites that play a central role in the biochemistry of the matrix, such as acetyl-CoA, FADH<sub>2</sub>, and NADH, actually exceed carboxyfluorescein in size, and since all metabolites must associate with and dissociate from

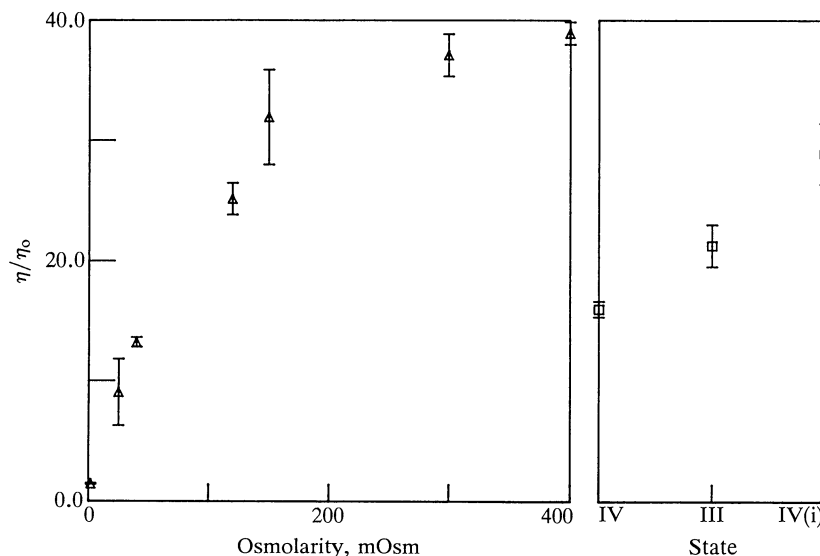


FIG. 4. Normalized effective viscosity vs. osmolarity and respiratory state. Experimental conditions were identical to those in Fig. 3.

enzymes, it is anticipated that the hindrance of motion that we have detected for the fluorophore may be even more pronounced for some matrix metabolites.

Our data also indicate that matrix viscosity is volume (or configuration) dependent. This observation too has potentially important biological implications. It appears that a number of matrix enzymes turn over more rapidly as the volume of the matrix increases in response to hormonal stimuli (23, 24). If metabolite diffusion is involved in the mechanism of action of these enzymes, then such enhanced turnover could reflect more rapid diffusion of metabolites that is brought about by a reduction in viscosity as matrix volume increases. (We note, however, that the issue at hand is not trivial to analyze since metabolites must also on average diffuse farther at larger matrix volumes.)

The above analysis should be contrasted with suggestions that metabolism in the matrix involves the directed (nondiffusive) channeling of substrates from one enzyme to another in a sequence (for review, see ref. 4). To date, it has been difficult to demonstrate that channeling occurs in the matrix. However, this hypothesis is supported by studies of urea cycle enzymes that indicate that extramitochondrial ornithine is channeled between its transporter and ornithine transcarbamylase (25) and by studies of certain tricarboxylic acid cycle reactions that have been shown to maintain an initially asymmetric <sup>13</sup>C-labeling pattern (26, 27). Our diffusion measurements also bear on the existence of channeling in the matrix. If the diffusion of small molecules were completely inhibited in the crowded matrix environment, one might suspect that channeling occurs. However, since we find that our probe rotates slowly in the matrix but is not immobile, channeling (although it may occur) is probably not an essential consequence of a crowded matrix environment. Measurement of an effective translational viscosity for the matrix would further help to resolve this issue.

**Implications for Structure in the Matrix.** It is also of interest to discuss how the anisotropy measurements bear on current ideas about the molecular organization of the matrix. It has been suggested that the matrix has a network-like structure (7). It has also been suggested that the matrix is a dense-packed protein solution in which the average diameter of protein-free aqueous pores is only 2 nm (28) and in which water has very nonideal properties (29, 30). For example, it appears that matrix water is 3–4 times less mobile than bulk water (30) and could even exist in two phases—one “bound” to protein or membrane and highly viscous and the other “free” and with a viscosity equal to that of dilute buffer (29).

Our data support the general concept of a dense-packed matrix in which the space available for diffusive motion is limited. For example, this concept is consistent with our observation that diffusion is severely hindered in the aqueous phase of the condensed matrix and that diffusion accelerates as the matrix becomes orthodox and its water content and pore size increase. Further support for this hypothesis comes from the existence of a maximum plateau value for the anisotropy at osmolarities  $\geq 300$  mOsm (Fig. 3), a result that suggests that the matrix protein in a condensed mitochondrion is so tightly packed that further structural change is unlikely to occur. Of course, the high viscosities (and slow diffusion) detected here could arise either as a “weighted” contribution from two distinct aqueous phases, such as

bound and free water with relatively higher and lower viscosities (29), or from a single aqueous phase that actually has the measured viscosity. Time-resolved anisotropy measurements should distinguish between these two possibilities. Whatever the exact structure of the matrix may be, it is now becoming clear that molecular dynamics, structure, and function in the mitochondrial matrix are very profoundly influenced, and perhaps modulated, by variations in matrix protein concentration.

We thank Steve Burgess, Jogen Wu, and Barry Lentz for their assistance in measuring the lifetime of carboxyfluorescein; Brad Chazotte, Jorge Cortese, Charlene Dickerson, Jeremy Foy, Ian Heath, Patrick Igwenagu, and Laura Voglino for helpful conversations and assistance with the isolation of mitochondria; Arthur Palmer, Mary Lee Pisarchick, and Nancy Thompson for assistance with Figs. 3 and 4; and Yukio Tanaka for assistance with electron microscopy. This work was supported by National Science Foundation Grant PCM 88-16611 and National Institutes of Health Grant GM 28704.

1. Kellenberger, E. (1987) *Trends Biochem. Sci.* **12**, 105–107.
2. Srere, P. A. (1980) *Trends Biochem. Sci.* **5**, 120–121.
3. Srere, P. A. (1982) *Trends Biochem. Sci.* **7**, 375–378.
4. Srere, P. A. (1987) *Annu. Rev. Biochem.* **56**, 89–124.
5. Hackenbrock, C. R. (1966) *J. Cell Biol.* **30**, 269–297.
6. Hackenbrock, C. R. (1968) *J. Cell Biol.* **37**, 345–369.
7. Hackenbrock, C. R. (1968) *Proc. Natl. Acad. Sci. USA* **61**, 598–605.
8. Stoner, C. L. & Sirak, H. D. (1969) *J. Cell Biol.* **43**, 521–538.
9. Haugland, R. P. (1989) *Molecular Probes Handbook of Fluorescent Probes and Research Chemicals* (Molecular Probes, Eugene, OR), pp. 89 and 105.
10. Davis, M. H., Altschuld, R. A., Jung, D. W. & Brierley, G. P. (1987) *Biochem. Biophys. Res. Commun.* **149**, 40–45.
11. Reers, M., Kelly, R. A. & Smith, T. W. (1989) *Biochem. J.* **257**, 131–142.
12. Mastro, A. M. & Keith, A. D. (1984) *J. Cell Biol.* **99**, Suppl., 180s–187s.
13. Drake, J. M. & Klafter, J. (1990) *Phys. Today* **43**(5), 46–55.
14. Cantor, C. R. & Schimmel, P. R. (1980) *Biophysical Chemistry* (Freeman, San Francisco), pp. 459–466.
15. Lakowicz, J. R. (1983) *Principles of Fluorescence Spectroscopy* (Plenum, New York), pp. 111–153.
16. Shinitzky, M., Dianoux, A.-C., Gitler, C. & Weber, G. (1971) *Biochemistry* **10**, 2106–2113.
17. Schneider, W. C. (1948) *J. Biol. Chem.* **176**, 259–266.
18. Estabrook, R. W. (1967) *Methods Enzymol.* **10**, 41–47.
19. Hackenbrock, C. R. (1972) *J. Cell Biol.* **53**, 450–465.
20. Chance, B. & Williams, G. R. (1955) *J. Biol. Chem.* **217**, 409–427.
21. Hackenbrock, C. R. & Miller Hammon, K. (1975) *J. Biol. Chem.* **250**, 9185–9197.
22. Dix, J. A. & Verkman, A. S. (1990) *Biophys. J.* **57**, 231–240.
23. Halestrap, A. P. (1989) *Biochim. Biophys. Acta* **973**, 355–382.
24. Watford, M. (1990) *Trends Biochem. Sci.* **15**, 329–330.
25. Cohen, N. S., Cheung, C. & Rajjman, L. (1987) *J. Biol. Chem.* **262**, 203–208.
26. Sumegi, B., Sherry, A. D. & Malloy, C. R. (1990) *Biochemistry* **29**, 9106–9110.
27. Srere, P. A. (1990) *Trends Biochem. Sci.* **15**, 411–412.
28. Srere, P. A. (1981) *Trends Biochem. Sci.* **6**, 4–7.
29. Garlid, K. D. (1979) in *Cell-Associated Water*, eds. Drost-Hansen, W. & Clegg, J. (Academic, New York), pp. 293–361.
30. Srere, P. A., Sumegi, B. & Sherry, A. D. (1987) *Biochem. Soc. Symp.* **54**, 173–181.