*Journal of Bioenergetics and Biomembranes, Vol. 29, No. 6, 1997*

# Localized Firefly Luciferase Probes ATP at the Surface of Mitochondria

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*Received July 14, 1997*

The concentration of ATP generated by yeast mitochondria and consumed by yeast hexokinase was monitored using native firefly luciferase in solution, or recombinant luciferase localized at the surface of mitochondria. In the absence of hexokinase, both probes perform similarly in detecting exogenous or mitochondrially-generated ATP. The steady-state concentrations of ATP can be reduced in a dose-dependent manner by hexokinase. With hexokinase added in large excess, the localized probe reports substantial ATP concentrations while none is detectable by soluble luciferase. Thus, ATP accumulates near the membrane where it appears, relatively to solution, and vice versa for ADP. The extent of nucleotide gradients is shown to be correlated with the specific activity of oxidative phosphorylation and with the viscosity of the medium, but independent of the concentration of the organelles. A simple model involving diffusional restrictions is presented to describe this behavior. The metabolic and evolutionary implications of cellular catalysis limitation by physical processes are discussed.

**KEY WORDS:** Luciferase; localized probe; heterogeneous coupled systems; mitochondria; hexokinase; nucleotide concentration gradients; cellular catalysis.

## INTRODUCTION

Cellular metabolic processes *in situ* are highly coordinated through elaborate control mechanisms. In the standard analysis of cellular catalysis, homogeneity of the internal environment is often implicitly assumed, in flagrant contradiction with the high degree of organization in cells (Clegg, 1984; Porter, 1987). Thus, metabolites, effectors, as well as cytoplasmic enzymes and organelles are often treated as rapidly diffusible, homogeneously distributed components. Such an approximation precludes accounting for essential means of metabolic control via a dynamic (re)arrangement of catalytic units achieved through specific macromolecular interactions (Keleti *et al.*, 1988; Masters, 1981; Srere, 1987). A critical appraisal of the role for enzyme location and the involvement of physical processes in cellular metabolism requires the assessment of local concentrations of metabolites in the intricate intracellular milieu. Hence, localized processes could only be assessed through indirect experimental evidence. Measurements with localized probes (Aflalo, 1991; Aflalo and DeLuca, 1987; Jones and Aw, 1990) should therefore contribute exclusive information on cryptic microscopic events, as opposed to the more readily assessed macroscopic fluxes, and help to better understand the structural basis of cellular function.

Soluble firefly luciferase (FL[2]) can specifically monitor ATP in solution with a high sensitivity (DeLuca and McElroy, 1978). Thus, light emission from a localized enzyme should report the concentration of ATP in its immediate vicinity. The first direct measurements of local [ATP] were conducted in artificial model systems, with FL covalently immobilized within Sepharose beads, in the presence of coimmobi-

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[2] Abbreviations: BSA: bovine serum albumin; DTT: dithiothreitol; EDTA: ethylene diamine tetraacetic acid; FL: firefly luciferase; G6PDH: glucose-6-phosphate dehydrogenase; Hepes: 2-[4-(2-hydroxyethyl-1-piperazine)] ethanesulfonic acid; HK: hexokinase; IU: international unit of enzyme activity (= 1 μmol product/min); PK: pyruvate kinase; PMSF: phenylmethylsulfonyl fluoride; WT: wild type (mitochondria).



0145-479X/97/1200-0549$12.50/0 

lized enzymes producing or consuming ATP (Aflalo and DeLuca, 1987). In these studies the contribution of diffusion to both local and macroscopic measurements has been mathematically modeled, and the importance of physical steps in heterogeneous systems has been demonstrated on a quantitative basis.

A more realistic reconstituted biological system consisting of isolated mitochondria coupled to the hexokinase (HK) reaction was designed to assess [ATP] at steady state using differently localized probes. The luciferase gene has been genetically engineered so that the luminescent product expressed in yeast is targeted and anchored to the outer membrane of mitochondria (Aflalo, 1990). The basic principles for the dual use of localized and soluble luciferase as probes for ATP measurements in biological systems have been outlined (Aflalo, 1991). Based on preliminary results obtained with the mitochondria–hexokinase coupled processes, the behavior of such hetero-geneous cyclic systems has been mathematically modeled for various operating conditions (Aflalo and Segel, 1992).

This communication focuses on the experimental aspects of macroscopic and local [ATP] measurements in the mitochondria–hexokinase system using soluble and localized firefly luciferase, respectively. The results are analyzed in terms of a simple two-compartment system. The experimental data demonstrate the occurrence of concentration gradients of nucleotides at steady state in reconstituted coupled systems. The analysis of the results in light of the proposed model provides a powerful tool for a better characterization of real bioenergetically-coupled systems based on conventional measurements.

## MATERIALS AND METHODS

Soluble firefly (*P. pyralis*) luciferase, baker's yeast hexokinase, rabbit muscle pyruvate kinase (PK), bovine pancreas trypsin, soybean trypsin inhibitor, and *Leuconostoc* glucose-6-phosphate dehydrogenase (G6PDH), as well as D-luciferin, ATP, ADP, and dextran (average $M_r$ = 40,000) were from commercial sources.

### Biological Materials

The construction of a fusion gene between *MAS70* [encoding for a *S. cereviciae* 70-kDa mitochondrial protein (Reizman *et al.*, 1983)] and *luc* [encoding for FL (de Wet *et al.*, 1985)] as well as its insertion in the yeast–bacteria shuttle plasmid pFL1 has been described (Aflalo, 1990). The N-terminal moiety (93 residues) of the chimeric product includes the sequence which targets and anchors the parent mitochondrial protein to the cytoplasmic face of the mitochondrial outer membrane (Hase *et al.*, 1984; Reizman *et al.*, 1983). The product, retained on mitochondria, is endowed with bioluminescence activity in intact yeast, as well as in cell-free extracts. The yeast strain DL1 transformed with the host plasmid pFL1 was used for the isolation of wild type (WT) mitochondria. The same strain transformed with the plasmid pFL1-CA (Aflalo, 1990) harboring the engineered FL fusion gene was used as a source of mitochondria with bound FL.

### Preparation of Yeast Mitochondria

Mitochondria were isolated from exponential yeast cultures growing aerobically on lactate as described (Aflalo, 1990; Hase *et al.*, 1984). Briefly, yeast spheroplasts were prepared and homogenized, and mitochondria isolated by three cycles of differential centrifugation in an isosmotic buffer (Buffer A: 0.6 M sorbitol, 50 mM K-Hepes pH 7.8, 20 mM KCl) supplemented with protective reagents (1 mM DTT, 1 mM PMSF, 1 mM K-EDTA, and 1 mg/ml BSA). The final mitochondrial pellet was resuspended in Buffer A at a protein concentration of 20 mg/ml and used immediately or rapidly frozen and stored at –70°C for up to one month with some loss of phosphorylative activity (20–30%) but stable FL activity when present.

### Luminometric Assays

FL activity was assessed at 25°C by measuring the light produced in the presence of saturating concentrations of substrates. Small aliquots containing mitochondria and FL were added to a reaction mix (0.1 ml) containing 0.6 M sorbitol, 20 mM K-Hepes pH 7.8, 1 mM K-EDTA, 4 mM Mg-Hepes, 1 mM ATP, and 1 mg/ml BSA, inserted and stirred in the chamber of an LKB 1250 luminometer. After the automated rapid injection of luciferin (0.2 mM in 0.1 ml of the same medium), a characteristic peak of light is recorded whose maximal intensity (in volts) is proportional to FL activity (DeLuca and McElroy, 1978). In some measurements, BSA was replaced by 0.5% Triton X-100 in the reaction mix. The measurements were made in triplicate.

Continuous luminometric monitoring of ATP evolution by soluble (Lemasters and Hackenbrock, 1979) or mitochondria-bound (Aflalo, 1990) FL was performed by recording the light output which is linearly related to low ATP concentrations (up to 2 μM). Under these conditions, the bioluminescence intensity is proportional to both the amount of FL and ATP concentration (Aflalo, 1991). The basic reaction mix was as described above except that 0.1 mM luciferin, but no ATP, was included. The light signal from soluble or bound FL was calibrated using the increase in light output following the addition of a standard ATP solution. The concentrations of ADP and ATP in stock and standard solutions were calculated from light absorbance at 259 nm; their purity (<5% cross-contamination) was assessed enzymatically using pyruvate kinase in bioluminescent assays with FL.

### Coupled System Assays

Wild type mitochondria supplemented with soluble FL, or alternatively mitochondria with bound FL (from recombinant yeast), were suspended in a stirred reaction mix (0.5 ml) containing in addition 5 mM K-$P_i$, 10 mM K-succinate, and 5 mM glucose. The endogenous adenine nucleotide content and the rate of oxidative phosphorylation (observed after the addition of mitochondria and ADP, respectively) were calculated using the calibration performed as described above. In some instances, we used pyruvate kinase and phosphoenol pyruvate (1 mM) as a soluble ATP-generating system instead of mitochondria-succinate-$P_i$. In both systems the extent of ADP to ATP conversion was routinely calculated (typically 85–105%). Finally, the concentration of ATP in each experiment was also reduced by successive additions of yeast HK (up to 32 IU/ml) to reach progressively reduced steady states.

The steady-state rate of phosphorylation at 1 μM ADP in each system (PK, WT, and recombinant mitochondria) tested in spectrophotometric assays using excess HK (1 IU/ml) coupled to G6PDH (2 IU/ml, supplemented with 1 mM $NADP^+$) in identical conditions was found similar to that observed in the corresponding continuous measurements with either bioluminescent probe.

## RESULTS

Light measurements with intact cells expressing cloned FL (Aflalo, 1990; de Wet *et al.*, 1987; Gandelman *et al.*, 1994; Vieites *et al.*, 1994) indicate that intracellular ATP is saturating. Thus, straightforward local ATP measurements are possible only with reconstituted systems for which a strict control of the conditions for the operation of the probe (exposed to the medium) and its calibration (at low [ATP]) can be achieved.

### Localization of Native and Targeted Luciferase

The location of soluble FL added to WT mitochondria, or that of the chimeric FL retained on isolated recombinant organelles, as well as their respective distribution between the bulk medium and mitochondrial compartments, may greatly influence the quality of ATP measurements. It is essential to ascertain the exclusive localization of each probe in its own compartment. This has been assessed by following their respective bioluminescence activity during proteolysis. Figure 1 shows the time course (A) and distribution (B) of residual enzymatic activity from soluble and engineered FL upon trypsin treatment in the presence of mitochondria. While the purified FL does not associate with WT mitochondria and it is relatively insensitive to trypsin, the fully mitochondria-associated fusion product is first clipped off, releasing all the active FL moiety to the medium, and further completely des-

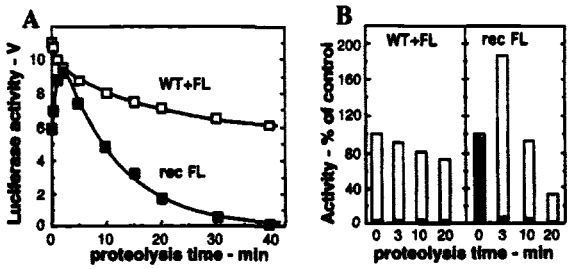



**Fig. 1.** Trypsin treatment of soluble and mitochondria-bound luciferase. A: Mitochondria (1 mg/ml) isolated from wild type (open symbols) or transformed (closed symbols) yeast cells were incubated on ice in Buffer A with trypsin (10 μg/ml). WT mitochondria were supplemented with soluble FL (10 ng/ml). At the indicated times, soybean trypsin inhibitor was added in excess (0.2 mg/ml) and aliquots from the suspension were analyzed for FL activity. Further treatment of fully digested samples with neutral detergent (0.5% Triton X-100) yielded no detectable activity. B: Localization of residual activity. Samples from the wild type (left) or transformed (right) mitochondrial suspensions in A were fractionated by centrifugation (13,000 *g*, 10 min) and then analyzed for FL activity in the soluble (open) and particulate (closed) fractions.

troyed by the proteolytic enzyme. After the bioluminescent moiety has been completely released (3 min) or digested (45 min), the presence of residual FL in detergent-solubilized mitochondrial pellets was tested, yielding no detectable activity (not shown). This indicates that no additional enzyme is present in internal compartments (inaccessible to trypsin).

Thus, while the purified enzyme occurs exclusively in solution, the fusion protein seems to be specifically located at the cytoplasmic face of the outer membrane of yeast mitochondria and should therefore be fully accessible to both ATP added to the medium (and diffusing to the membrane, in the absence of consuming processes) and that emerging from mitochondria.

## Local Versus Bulk [ATP] Measurements

Figure 2 presents typical luminometric traces for ATP evolution in three basic systems in which ATP was generated by PK in solution (a), or by oxidative phosphorylation in WT (b) or recombinant (c) mitochondria; it was monitored by soluble (a,b) or membrane-bound (c) FL. With the addition of mitochondria to the reaction mix containing the substrates of oxidative phosphorylation, a small increase in light intensity is observed. This indicates that endogenous nucleotides—retained even after extensive washing of the organelles—are phosphorylated and released to the medium. The calibration of both the localized and soluble FL using exogenous ATP was performed similarly. When ATP formation is initiated, one cannot distinguish between the three systems in terms of the kinetics of phosphorylation. In these conditions where ATP appears slowly in the medium and is not consumed, the heterogenous systems behave as if they operated in a homogenous solution.

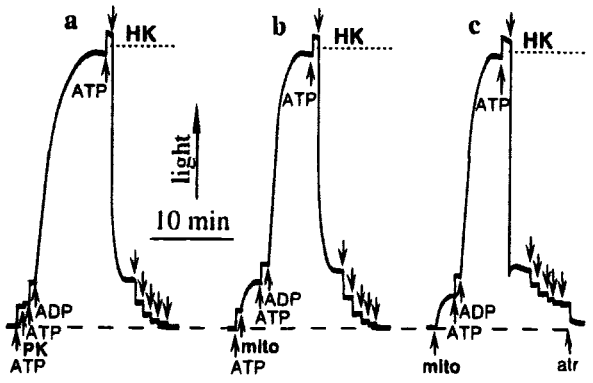



**Fig. 2.** Light emission from soluble (a, b) or localized (c) luciferase during ATP formation coupled to HK in solution. The reaction conditions were as described in Materials and Methods. ATP formation was catalyzed by (a) pyruvate kinase (PK, 100 mIU/ml) in the presence of 1 mM phosphoenol pyruvate (instead of $P_i$ and succinate), (b) WT, or (c) recombinant mitochondria (mito, 40 μg/ml). Soluble FL (10 ng/ml) was added to PK and WT mitochondria reactions in (a and b). The indicated additions (in small volume) were 0.1 μM ATP, 1 μM ADP, and 10 μg/ml atractyloside (atr). At the downward arrows, yeast HK was added sequentially from serial dilutions so that its concentration doubled each time (1–32 IU/ml).

In all three cases, ATP formation was further coupled to its consumption in solution by added HK. The first system (Fig. 2a) in which ATP is generated, monitored, and consumed in solution reflects the conditions of regular homogenous catalysis. The latter cases with ATP appearing at the surface of mitochondria and consumed in solution represent two heterogenous systems in which ATP is monitored either in solution (Fig. 2b) or in close vicinity to the mitochondrial outer membrane (Fig. 2c). In the control systems (Figs. 2a & b), the steady state [ATP] can be reduced to undetectable concentrations at high enough HK. However, the light output from mitochondria-bound FL is reduced to a finite value (Fig. 2c) under similar conditions. This value could be lowered further only in the presence of mitochondrial inhibitors like atractyloside (Fig. 2c) and other inhibitors of oxidative phosphorylation which do not affect the luciferase activity (not shown). The residual ATP measured with inhibited mitochondria reflects the contribution of adenylate kinase in the inter-membrane space to ATP formation.

A graphical approach may be used to assess the theoretical lower limit for ATP in the presence of excess HK. The data on [ATP] presented in Fig. 2 are replotted as a function of the reciprocal of HK activity (1/[HK]). The plots shown in Fig. 3 indicate that this function becomes quasi-linear in the high [HK] range. With the membrane-bound probe, the finite value for the extrapolation to the ordinate axis at a zero value for the abscissa (infinite [HK]) represents the ATP concentration accumulated at steady state near the outer membrane while it is completely depleted in solution. This value will be further referred to as $[ATP]_{extp}$.

The results indicate that when ATP is efficiently depleted in the medium by HK (as detected by soluble FL), the ATP emerging from the mitochondria is still available to the localized probe. Thus, at least for high

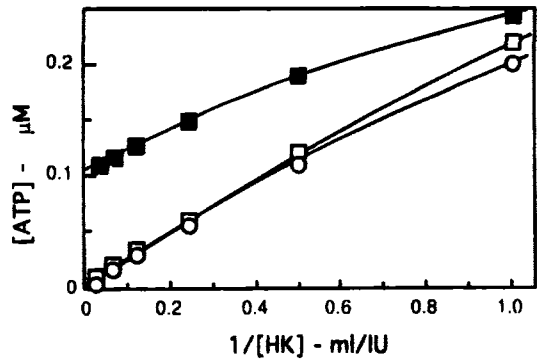



**Fig. 3.** Graphical derivation of ATP concentrations at steady state during its formation in the presence of infinite HK activity in solution. The data represent replots of the results in Fig. 2. ATP generated by PK (circles) or mitochondria (squares) was monitored either in solution (open symbols) or at the surface of mitochondria (closed symbols).

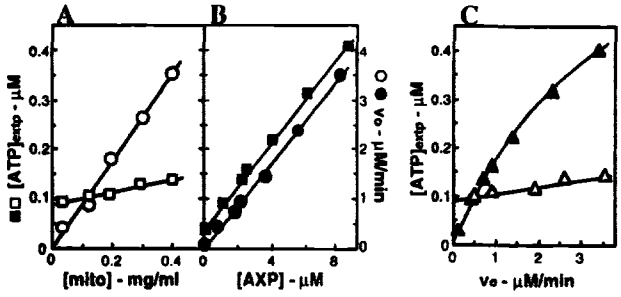



**Fig. 4.** Dependence of ATP accumulation at the surface of mitochondria on the rate of phosphorylation. The experiments with recombinant mitochondria were performed as in Fig. 2c, except for the indicated variation of mitochondria and added ADP. The original data were processed as explained in the text. The derived values for the initial velocity of oxidative phosphorylation ($v_o$, circles) and for steady-state [ATP] extrapolated to infinite [HK] ($[ATP]_{extp}$, squares) in panels A and B were plotted against the concentrations of added recombinant mitochondria ([mito], with 1 µM ADP) or nucleotides ([AXP], with 40 µg/ml mitochondria), respectively. In panel B $v_o$ is plotted against added [ADP], and $ATP_{extp}$ is plotted against the total concentration of adenine nucleotides present in each experiment. The latter was taken as the ATP concentration measured immediately before the first addition of HK (see Fig. 2), and it included exogenous ADP and ATP as well as the endogenous nucleotides added together with mitochondria (2.8 nmol/mg). Replots of the data in A (mito varied, open symbols) and B (ADP varied, closed symbols) are presented in panel C.

catalytic rates of ATP consumption in solution, the coupled heterogeneous system may be limited by diffusion of nucleotides between the surface of mitochondria and the bulk solution.

## Dependence on the Rate of ATP Formation

The extent of the actual limitation by diffusion in the coupled system is expected to be inversely related to the catalytic ability of the concurrent enzymic steps (Engasser and Horvath, 1976). Thus, upon increasing the specific activity of phosphorylation, more ATP should accumulate at steady state near the outer membrane, as is suggested by the opposite effect obtained by inhibiting phosphorylation with atractyloside (Fig. 2c). On the other hand, an increase in the concentration of mitochondria in the system, while increasing the overall rate for ATP appearance in the bulk medium, does not affect the flux of ATP emerging from the membrane (constant specific rate of phosphorylation). In this case, the local accumulation of ATP should not be affected. This rationale is validated by the data presented in Fig. 4.

A series of experiments was carried out with mitochondria bearing the localized probe essentially as described in Fig. 2c, but differing in the amounts of added mitochondria (Fig. 4A) or ADP (Fig. 4B). Two independent variables were derived from each resultant luminometric trace: (i) the initial velocity of oxidative phosphorylation ($v_o$) was calculated from the slope observed after addition of ADP; (ii) the finite extrapolated limit ($[ATP]_{extp}$) at infinitely fast ATP consumption in the medium was determined from replots of steady-state concentrations of ATP at variable [HK] (see Fig. 3). This limit represents the net accumulation of ATP near the membrane when its concentration in the bulk medium is zero.

The rate of oxidative phosphorylation assessed with membrane-bound luciferase follows first-order kinetics with respect to both mitochondria and ADP in the ranges tested. An identical behavior was observed with WT mitochondria and the soluble probe (not shown). However, while the relation between $[ATP]_{extp}$ and the total nucleotides concentration is nearly linear (Fig. 4B), the extrapolated value depends only marginally on the concentration of mitochondria (Fig. 4A). A quantitative comparison of both sets indicates that the linear increase in $[ATP]_{extp}$ (beyond the value extrapolated to zero mitochondria) can be attributed solely to the contribution of endogenous nucleotides (added together with mitochondria). Indeed, the latter increases the total nucleotide concentration present in the reaction mix, and thus tends to enhance the local accumulation of ATP (as shown in Fig. 4B). Thus, an increase in

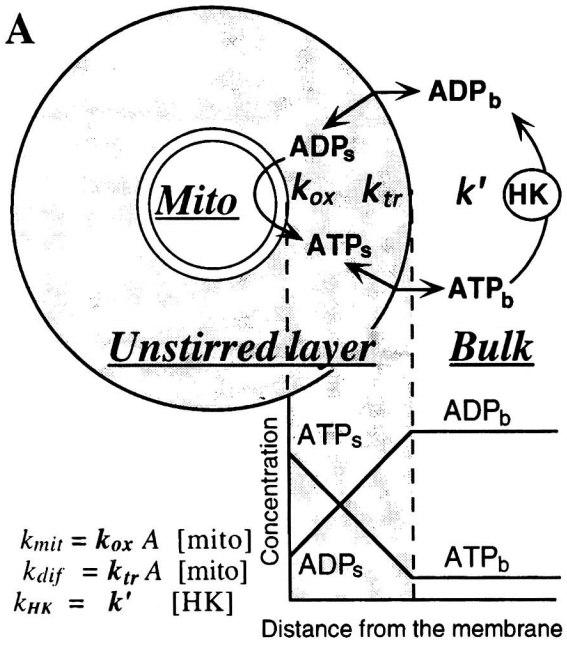
mitochondria concentration by itself does not increase the accumulation of ATP near the membrane. Under similar conditions (mitochondria and ADP concentrations), control experiments with WT mitochondria and soluble FL yielded values of zero for the extrapolation (Aflalo, 1991).

A replot of the data as $[ATP]_{extp}$ vs. $v_o$ (Fig. 4C) emphasizes the relation between ATP accumulation and the rate of oxidative phosphorylation in both experiments. This comparison is rather coarse since in the presence of excess HK the rate of phosphorylation at steady state is in fact higher than the value $v_o$ measured as the initial rate at 1 μM ADP, and it does include the contribution of endogenous nucleotides. Nevertheless, ATP accumulated at steady state is better correlated with the specific activity (when [ADP] is varied) than with the total activity (when mitochondria concentration is varied) of oxidative phosphorylation.

In summary, the accumulation of ATP near the membrane depends on its flux through the membrane (i.e., per unit area) rather than on the total rate of its appearance in the bulk medium, indicating that a true local event is being monitored by the membrane-bound probe.

## Modeling the Coupled Heterogeneous System

Such a behavior can be simulated by reducing the system to a simple model including two catalytic reactions coupled through diffusion of intermediates. Oxidative phosphorylation is modeled as a single catalytic step located at the surface of particles in suspension. Under the conditions used in this work, the initial rate of ATP formation depends linearly on the concentrations of both mitochondria and ADP, the dephosphorylation of ATP by HK in the bulk solution is similarly related to [ATP] and [HK]. Diffusion of nucleotides between the bulk medium and the surface can be approximated to a mass transfer process in an unstirred layer. The model is outlined schematically, and the pseudo-first-order rate constants for each process (independent of the nucleotides concentra-




B

$$\frac{d[ADP]_s}{dt} = k_{dif}([ADP]_b - [ADP]_s) - k_{mit}[ADP]_s \quad (1)$$

$$\frac{d[ATP]_s}{dt} = k_{mit}[ADP]_s - k_{dif}([ATP]_s - [ATP]_b) \quad (2)$$

$$\frac{d[ATP]_b}{dt} = k_{dif}([ATP]_s - [ATP]_b) - k_{HK}[ATP]_b \quad (3)$$

$$\frac{d[ADP]_b}{dt} = k_{HK}[ATP]_b - k_{dif}([ADP]_b - [ADP]_s) \quad (4)$$

$$[ATP]_b + [ADP]_b = [ATP]_s + [ADP]_s = [AdN] \quad (5)$$

$$\frac{[ADP]_s}{[ADP]_b} = \frac{k_{dif}}{k_{dif} + k_{mit}} = \frac{k_{tr}}{k_{tr} + k_{ox}} \quad (6)$$

$$\frac{[ATP]_s}{[ATP]_b} = \frac{k_{dif} + k_{HK}}{k_{dif}} \quad (7)$$

$$v_o = k_{obs}[ADP]_b = \left(\frac{1}{k_{dif}} + \frac{1}{k_{mit}}\right)^{-1}[ADP]_b \quad (8)$$

$$\frac{[ATP]_s^{extp}}{[AdN]} = \frac{k_{mit}}{k_{mit} + k_{dif}} = \frac{k_{ox}}{k_{ox} + k_{tr}} \quad (9)$$

**Fig. 5.** Model for coupling two catalytic steps in a heterogeneous system. A: Schematic model. The indices s and b for nucleotide species represent surface and bulk respectively. $k_{ox}$ (cm/min), $k_{tr}$ (cm/min), and $k'$ (ml.lU/min) are *intrinsic* second-order rate constants for oxidative phosphorylation, mass transfer at the surface, and glucose phosphorylation in solution. *A* is the specific area of mitochondria ($cm^2$/mg). The *effective* pseudo-first-order rate constants ($min^{-1}$) inferred for oxidative phosphorylation, mass transfer, and the HK reaction are denoted $k_{mit}$, $k_{dif}$, and $k_{HK}$, respectively. B: Phenomenological equations and steady-state solutions. Equations (1)–(4): rate laws for each nucleotide species; Eq. (5) general mass conservation; Eqs. (6)–(7): nucleotide concentration ratio surface:bulk at steady state [from Eqs. (1) and (3)]; Eq. (8): steady-state rate of ATP appearance at the surface ([HK] = 0); Eq. (9): steady-state solution for infinite [HK] (i.e., $[ATP]_b = 0$).

tion) are given in Fig. 5A. The equations describing the system are formulated in Fig. 5B, based on the following assumptions: (i) ADP and ATP diffuse identically, and (ii) the mass transfer in both directions is proportional to the difference in concentration of the transferred nucleotide between the surface and the medium, as well as to the total area. To simplify further, it was also assumed that the nucleotides diffuse in a near-planar unstirred layer sticking to the mitochondrial surface (devoid of catalytic activities) along a linear gradient, and the volume of both mitochondria and the diffusion layer is negligible compared to that of the bulk medium.

In the absence of HK, the effective rate for phosphorylation of added ADP by mitochondria (as detected at the surface) may be represented by a single process with a composite pseudo-first-order rate constant [$k_{obs}$, Eq. (8)] combining the sequential mass transfer and surface catalysis. This value, proportional to the concentration of mitochondria, can be determined experimentally as the slope of the observed initial velocity of phosphorylation vs. added [ADP] (0.45 $min^{-1}$ in Fig. 4B).

In the presence of HK, one can solve the system of differential equations for steady state, and by introducing [AdN] as the sum of endogenous and added nucleotides [mass conservation, Eq. (5)], a simple expression for the local [ATP] at infinite HK concentration (at which [AdN] = $[ADP]_b$) can be reached. Since both $k_{mit}$ and $k_{dif}$ are proportional to the concentration of mitochondria, it results that the fraction of ATP accumulated at the surface is proportional to [AdN] and independent of [mito] [Eq. (9)], in agreement with the results in Fig. 4C. The ratio $k_{mit}/k_{mit} + k_{dif}$ can thus be determined as the slope of $[ATP]_{extp}$ vs. [AdN] (0.045) in Fig. 4B.

One can also solve the system of equations (8)–(9) as formulated above for the pseudo-first-order rate constants. It turns out that mass transfer ($k_{dif}$ = 10 $min^{-1}$) is about 20 times faster than phosphorylation ($k_{mit}$ = 0.47 $min^{-1}$), so that it does not contribute significantly to rate limitation in the absence of HK. The ratio between the rates of mass transfer and phosphorylation is independent of the total area of mitochondria. However, as the rate constant for HK in solution increases, diffusion becomes more limiting relative to this additional catalytic step. In this case, as the rate of HK becomes infinitely fast, the accumulation of ATP at the surface [Eq. (7)] increases asymptotically to the theoretical maximum $[ATP]_{extp}$ [as formulated in Eq. (9)]. Finally, it is worthwhile noting that while the concentration ratio of ATP [surface: bulk, Eq. (7)] depends on the relative amounts of HK and mitochondria, that for ADP [Eq. (9)] reflects exclusively parameters inherent to mitochondria and is independent of the efficiency of the ATP trap (HK) in solution.

## Other Factors Affecting Accumulation of ATP at the Surface

As predicted in the previous section, a reduction of the diffusivity of intermediates in the coupled system should increase the accumulation of ATP near the mitochondrial membrane. The mass transfer of small molecules in well-stirred aqueous media is considered to be a fast process whose rate can be lowered by either increasing the viscosity (or molecular crowding) of the solution, or by reducing the stirring power (Aflalo and DeLuca, 1987; Engasser and Horvath, 1976). In the former case, such a change may also affect the microscopic rates of the catalytic steps. The neutral polymer dextran has been shown to induce major structural changes in rat liver mitochondria (Wicker *et al.*, 1993), without affecting their inherent catalytic activities (Gellerich *et al.*, 1993).

The results in Table I present the effect of sorbitol and dextran on the accumulation of ATP in the coupled system, as well as on other parameters providing pertinent information on the structure and function of the mitochondria. Sorbitol is routinely used to provide a proper osmoticum for the isolation of mitochondria and their operation in suspension. Solutions containing sorbitol at isosmotic concentration (0.6 M) are moderately viscous, which may impede diffusion. When the sorbitol concentration was reduced, a significant decrease in ATP accumulation near the membrane was observed (A). Under the hypotonic conditions achieved, neither the activity of the membrane-bound luciferase nor the release of endogenous nucleotides were notably altered. The small decrease in the rate of phosphorylation observed with these swollen mitochondria cannot account for the lowered ATP accumulation (see Fig. 4C).

As a control, mitoplasts were used instead of mitochondria (Table IB). These hypotonically treated particles are devoid of a sealed outer membrane and 60% of endogenous nucleotides were released and washed away. The mitoplasts nevertheless retained as much as 75% of the mitochondria-bound luciferase activity. This suggests that at least during the treat-

**Table I.** Effect of Viscosity in the Medium on the Retention of ATP at the Surface of Mitochondria[a]

| | Operating conditions | | | Net ATP accumulation[b] | Oxidative phosphorylation rate[c] | Luciferase activity[c] | Endogenous nucleotides |
|---|---|---|---|---|---|---|---|
| | Treatment | Sorbitol | Dextran | | | | |
| | | M | % (w/v) | μM | μM/min | mV/μM | nmol/mg |
| A | None | 0.6 | 0 | 0.092 | 1.02 | 20.2 | 2.0 |
| | None | 0.3 | 0 | 0.080 | 0.88 | 18.4 | 2.2 |
| | None | 0.0 | 0 | 0.065 | 0.90 | 19.8 | 1.8 |
| B | Mitoplasts | 0.6 | 0 | 0.060 | 0.66 | 15.2 | 0.8 |
| C | None | 0.6 | 0 | 0.082 | 0.86 | 16.1 | 1.8 |
| | None | 0.6 | 4 | 0.155 | 0.90 | 13.4 | 1.9 |
| | None | 0.6 | 8 | 0.189 | 0.67 | 12.6 | 2.0 |
| | None | 0.6 | 12 | 0.187 | 0.61 | 9.2 | 2.0 |

[a] Mitochondria (80 μg/ml) isolated from recombinant yeast were suspended in a reaction mix containing sorbitol and dextran (40,000 average $M_r$) as indicated, in order to vary the osmotic (A) or oncotic (C) properties of the medium, respectively. The samples were otherwise treated as described in Fig. 2c, and the indicated parameters derived from the corresponding luminometric traces. In experiment B, the mitochondria used in A were previously subjected to a hypotonic treatment, washed, and resuspended in isosmotic medium as described (Hase *et al.*, 1984), yielding mitoplasts which were used (80 μg/ml) instead of mitochondria.

[b] The limit of detected steady-state [ATP] at infinite [HK] (see $[ATP]_{extp}$ in Fig. 4).

[c] The activities for oxidative phosphorylation (at 1 μM ADP) and luciferase (from calibration).

ment, most of the enzyme is confined to areas of the outer membrane which are not torn away, possibly around sites of contact with the inner membrane. The mitoplasts can sustain a substantial rate of phosphorylation, which enables a significant ATP accumulation at steady state.

The effect of dextran at concentrations up to 12% (equivalent to about 3 mM) was also studied (Table IC). In this case the viscosity of the medium was increased by an order of magnitude [from 1.3 to 11 relative to water (Wolf *et al.*, 1977)], and special care was taken to achieve a proper dispersion of the various additions and ensure a steady mixing during the measurements. The main effect observed with added dextran in the mitochondria–HK coupled system is a strong increase in the ATP detected near the membrane at steady state in the presence of HK in excess. This change, reflecting a significant contribution of mass transfer as a rate limiting process, occurs while the apparent activity of oxidative phosphorylation is reduced in the presence of dextran. One can also notice a substantial inhibitory effect of dextran on the activity of the localized luciferase as revealed by its calibration. Separate controls indicated no influence of dextran on the HK activity, while only a mild reduction was observed with luciferase in solution. The effect on endogenous nucleotides released upon activity is only marginal.

In the presence of excess HK, the concentration of ATP measured at steady state is utterly independent of mixing (not shown), indicating that under the hydrodynamic conditions for the mitochondrial suspension (viscosity, particle dimensions, etc.), the stirring power we use is ineffective in reducing the width of a hypothetical diffusion layer around the mitochondria (Aflalo and Segel, 1992). This is indicative that diffusional restrictions are not confined to a restricted unstirred layer around the mitochondria, but they operate in fact over the whole space between mitochondria. Only at the highest dextran concentration was a small but significant increase in ATP accumulation observed upon stopping the stirring.

## DISCUSSION

The results presented above demonstrate that mitochondria-bound luciferase is able to monitor selectively the concentration of ATP present at the surface of mitochondria. This is done independently of the source of ATP, either exogenously added or released from internal compartments of the mitochondria. The results of local ATP measurements emphasize the difference with continuous monitoring of ATP in solution by soluble luciferase (Lemasters

and Hackenbrock, 1979), equivalent to other conventional analytic procedures which are unsuitable to reveal the heterogeneous behavior of cellular catalytic systems.

### Activity of the Localized Probe under Various Modes of Operation

A mild treatment of the chimeric mitochondria-bound probe with trypsin (Fig. 1) is able to release all detectable luciferase activity to the medium, similarly to what was found with the parent outer membrane protein contributing the N-terminal sequence (Reizman *et al.*, 1983), due to the presence of trypsin-sensitive sites in the linker between the anchor and the cytosolic domain. Further proteolytic treatment fully destroys the released luminescence activity. Moreover, no latent activity is uncovered by treatment with neutral detergent (Aflalo, 1990). This, together with the results obtained with mitoplasts (Table IB), conclusively rules out the presence of a hypothetical physical barrier between mitochondria-bound luciferase and the bulk solution.

Some notable differences in the sensitivity of luciferase variants to trypsin were observed. The most sensitive form is the luciferase domain after it was clipped off the membrane anchor by proteolysis (Fig. 1). Solubilization of the full-length chimeric protein by neutral detergent appears on the other hand to protect it against digestion by trypsin (Aflalo, 1990) to a similar extent as native luciferase in solution. This phenomenon may reflect a loose packing state of the N-terminal end of the clipped luciferase domain (a floppy end should be more accessible to the protease). The occurrence of the chimeric product in mixed micelles may result in a reduced exposure of trypsin-sensitive sites to the protease in solution, relatively to its membrane-anchored conformation.

A large apparent increase in activity is observed upon releasing luciferase to the medium by trypsin (Fig. 1), but not by detergent (Aflalo, 1990). Moreover, at limiting ATP concentrations the presence of dextran inhibits the activity of the membrane-bound (Table IC), but not that of the soluble probe (not shown). These facts suggest that the proximity of the membrane, rather than the presence of the N-terminal appendage in the engineered probe, affects the activity of the bound enzyme. The lower activity of the localized probe may reflect partitioning of reactants [repulsion of substrates and/or accumulation of inhibitory products (Lemasters and Hackenbrock, 1977)] near the membrane (Aflalo, 1991), relative to solution.

### Competency of the Engineered Luciferase as a Local Probe

While the inherent activity of the localized probe might differ from that observed in solution, the continuous monitoring of ATP under different states of operation of the mitochondria should remain consistent with calibration performed with exogenous ATP. The localized probe was routinely calibrated (see Fig. 2) while no ATP generation or consumption took place. The slow catalytic reaction of luciferase which probes ATP does not result in a significant consumption during the course of the measurement (Aflalo, 1991; Aflalo and DeLuca, 1987). In this case, limitation by the relatively fast mass transfer is expected to be negligible.

One may distinguish between two states of respiring mitochondria: (i) in the absence of ADP (static head); (ii) during active oxidative phosphorylation, whether it is coupled to the HK reaction or not. In the absence of HK, the phosphorylation of ADP added to the medium can be seen as a composite process including its transfer to the surface of mitochondria, its chemical transformation, and the transfer of ATP back to solution (see Fig. 5). All three steps are coupled and after a steady state is reached, the observed rates of phosphorylation reported by either the localized probe or that in solution are similar, so they can be treated as a global steady state rate within the experimental error.

When a fourth step of fast nucleotide recycling is introduced (by adding excess HK in solution), ATP is depleted both in the bulk medium and near the membrane, where it reaches constant—but different—concentrations. The influence of HK on the activity of luciferase cannot be assessed in these conditions, since no accurate calibration can be performed at this stage (fast depletion by HK of added ATP). It is yet extremely improbable that HK has a positive influence on the activity of the membrane-bound luciferase, as compared to the soluble probe, at least to the extent observed (finite vs. zero values, respectively) with excess HK.

To conclude, the same procedure for calibration of the soluble or localized probe is adequate not only for a good estimation of the effective kinetic parameters for the mitochondria (which only marginally reflect the relatively fast mass transfer processes), but also for diagnosing the limitation imposed by mass transfer of nucleotides between the surface (ATP appearance and ADP depletion) and the solution (ADP appearance and ATP depletion).

### Relation of the Proposed Model to Physical Reality

The two-compartment model involving a thin unstirred layer represents an oversimplification of the heterogeneous coupled systems investigated. It is nevertheless useful to describe semiquantitatively most features in the behavior of the systems, as reflected using either global or localized means of observation. The establishment of a concentration gradient of nucleotides near the mitochondria, as well as its dependence on the catalytic activity of both mitochondria and HK, are predictable by the model. Moreover, estimates for kinetic constants inherent to the heterogeneous mitochondrial system (e.g., $k_{mit}$, $k_{dif}$) can be derived using the model from the experimentally determined effective (or macroscopic; e.g., $k_{obs}$) parameters. The former provides in turn the basis for the assessment of microscopic constants (e.g., $k_{ox}$, $k_{tr}$). However, in the light of this model, a variation in the rate of mass transfer, as observed in the presence of dextran, cannot be interpreted unequivocally. Indeed, a reduction in its microscopic rate constant may arise from a decrease in the diffusion constant ($D$) and/or an increase in the width ($\delta$) of the unstirred layer (assuming a linear gradient, $k_{tr} = D/\delta$). The lack of effect for stirring argues definitely against an accurate description of the system using the unstirred layer paradigm which otherwise provides an approximate but simple solution. A more elaborate model based on less assumptions (e.g., taking a nonplanar surface for mitochondria and allowing HK in the diffusion space) and which better accounts for the present experimental findings has been presented and thoroughly analyzed (Aflalo and Segel, 1992).

### Experimental Significance of ATP Accumulation at the Surface

The steady-state value of local [ATP] in the presence of excess HK represents 5–10% of the total nucleotide concentration added to isolated mitochondria. This value represents a lower limit for the true extent of diffusional restrictions since HK may selectively bind to porin on isolated outer membranes when added in large excess (Krause *et al.*, 1986), although such an association could not be observed *in situ* with mitochondria rapidly isolated from yeast (Kovac *et al.*, 1986). Any association of HK with the outer membrane of mitochondria would tend to dramatically reduce the observed accumulation of ATP at the surface (local reduction of formation and increase in consumption) (Aflalo and Segel, 1992). Finally, in the presence of dextran—which increases the viscosity of the medium and hence reduces the diffusion rate of solutes—the accumulation of ATP at steady state is greatly enhanced. With all facts and arguments taken together, the involvement of diffusional restrictions in this cyclic heterogeneous coupled system is conclusively demonstrated.

The reconstituted system used was tested at low nucleotide concentrations, as dictated by the calibration range for luciferase. This situation results in pseudo-first order conditions for all the catalytic systems, which consequently operate at maximal catalytic efficiency in terms of substrate utilization (Keleti *et al.*, 1988). This provides a higher sensitivity to detect kinetic limitation by physical steps in heterogeneous catalysis (Engasser and Horvath, 1976). Although ATP concentrations are far below these in the cellular environment (in the mM range, considered as "saturating"), ADP concentration in cells is much lower (tens of μM). In rapidly respiring mitochondria, depletion of ADP at the surface due to a slow diffusion relative to catalysis may thus represent a limiting factor for oxidative phosphorylation. In cells with high (aerobic) energy demand, this problem has been circumvented (Brdiczka, 1994) by bringing kinases [e.g., HK in brain (Wilson, 1995), or creatine kinase in muscle] in close proximity to mitochondria by specific interaction (mammalian HK with outer membrane) or localization (mitochondrial creatine kinase).

## CONCLUDING REMARKS

The experimental demonstration of diffusional restrictions in cellular systems opens the way to a new evaluation of the intermediary metabolism. Concentration gradients of nucleotides have been implicated in muscle (Saks *et al.*, 1994) and in other systems (Aflalo and Shavit, 1982; Arora and Pedersen, 1988; Aw and Jones, 1985; BeltrandelRio and Wilson, 1992; Laterveer *et al.*, 1994). The manifestation of metabolite gradients provides a sensible alternative to the common "dialysis bag" formalism used not only to describe, but often to decipher, the behavior of cellular heterogeneous systems by kinetic means, based on tools borrowed from homogeneous catalysis (McFarland *et al.*, 1994). The occurrence of physical factors modulating catalysis in cells represents an

important—but often underestimated—component to the driving force for the evolution of an organized metabolism featuring not only optimal catalytic efficiency but subtle regulation relying on its harmonious operation in a defined environment.

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