Metabolic Network Control of Oxidative Phosphorylation

MULTIPLE ROLES OF INORGANIC PHOSPHATE*

Salil Bose, Stephanie French, Frank J. Evans, Fredric Joubert, and Robert S. Balaban‡

From the Laboratory of Cardiac Energetics, NHLBI, National Institutes of Health, Department of Health and Human Services, Bethesda, Maryland 20892

Phosphate (Pi) is a putative cytosolic signaling molecule in the regulation of oxidative phosphorylation. Here, by using a multiparameter monitoring system, we show that Pi controls oxidative phosphorylation in a balanced fashion, modulating both the generation of useful potential energy and the formation of ATP by F$_{0}$F$_{1}$-ATPase in heart and skeletal muscle mitochondria. In these studies the effect of Pi was determined on the mitochondria [NADH], NADH generating capacity, matrix pH, membrane potential, oxygen consumption, and cytochrome reduction level. Pi enhanced NADH generation and was obligatory for electron flow under uncoupled conditions. Pi oxidized cytochrome b (cyto-b) and reduced cytochrome c (cyto-c), potentially improving the coupling between the NADH free energy and the proton motive force. The apparent limitation in reducing equivalent flow between cyto-b and cyto-c in the absence of Pi was confirmed in the intact heart by using optical spectroscopic techniques under conditions with low cytosolic [Pi]. These results demonstrate that Pi signaling results in the balanced modulation of oxidative phosphorylation, by influencing both $\Delta$G$_\text{H}$ generation and ATP production, which may contribute to the energy metabolism homeostasis observed in intact systems.

Phosphate (Pi)$^1$ is the substrate for the phosphorylation of ADP to ATP in oxidative phosphorylation. Because ADP and Pi are generated by ATPases in the cytosol, the potential roles of ADP and Pi as cytosolic feedback signaling molecules regulating the rate of ATP production was one of the first models of the cytosolic regulation of mitochondrial ATP production (1, 2).

However, over the years it has become apparent that the cellular regulation of oxidative phosphorylation is a very complex control network, with numerous potential rate-limiting steps affected by a variety of signaling molecules, including ADP, Pi, Ca$_{2}^{+}$, creatine, and Mg$_{2}^{+}$ (3–7). This network results in the ability of tissues to change significantly the rate of ATP generation without significantly modifying the metabolic intermediates coupled to many other processes in the cell. This has been termed an energy metabolism homeostasis (8). Toward a better understanding of this regulatory network, the effects of each putative signaling molecule on oxidative phosphorylation need to be characterized. The purpose of the current work was to further evaluate the effects of Pi on different regulatory sites of oxidative phosphorylation in cardiac mitochondria.

Phosphate is believed to enter cardiac mitochondria via a neutral phosphate transporter (Pi) in exchange for OH$^{-}$ or by co-transport with H$^{+}$ (9). Thus, Pi transport is linked to the mitochondrial inner membrane pH gradient ($\Delta$pH$_{i}$) and the phosphate concentration gradient but not to the membrane potential ($\Delta$Ψ). Although Pi transport has not been ascribed as a rate-limiting step for phosphate utilization in oxidative phosphorylation, this particular aspect of phosphate metabolism has not been extensively studied, especially in the intact cell conditions where the driving forces (matrix membrane gradient of pH and matrix [Pi]) are ill defined. With regard to the regulation of oxidative phosphorylation, Pi is a primary substrate for the phosphorylation of ADP by the F$_{0}$F$_{1}$-ATPase. In addition, Pi has also been implicated in modifying the free concentration of Mg$_{2}^{+}$ or Ca$_{2}^{+}$ ions, via chelation, resulting in the modulation of these ions effects on mitochondrial metabolic processes (10–12). Pi also has been shown to increase the mitochondria volume (13, 14) that could also lead to metabolic consequences (15). The mechanism for the volume increase with Pi is complex, although likely involves the osmotic load of matrix [Pi] together with the enhancement of $\Delta$Ψ (via dissipation of $\Delta$pH $\_i$ (16) through Pi activity) and electrohydrostatic uptake of K$^{+}$ (17). Pi has also been suggested to affect the mitochondrial transition pore (18). Phosphate has been shown to affect directly dehydrogenases and other enzymes involved in oxidative phosphorylation (19). The most notable effect was reported by Hansford and Chappell (20) who showed that Pi was obligatory for electron flow in isolated blowfly mitochondria. These potential multiple sites of action of Pi on oxidative phosphorylation suggest that Pi may play a much more complex role in this process than simply a substrate for ADP phosphorylation.

The purpose of this study was to evaluate the hypothesis that Pi regulates several sites in oxidative phosphorylation, in addition to its role as a substrate for the F$_{0}$F$_{1}$-ATPase. Specifically, we propose that Pi is one of the primary regulators of NADH generation. To test this hypothesis, isolated porcine

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‡ To whom correspondence should be addressed: Laboratory of Cardiac Energetics, NHLBI, National Institutes of Health, Department of Health and Human Services, Bldg. 10, Rm. BID-161, Bethesda, MD 20892.

1 The abbreviations used are: Pi, inorganic phosphate; $\Delta$G$_{H}$, proton motive force; cyto-b, cytochrome b; cyto-c, cytochrome c; Pi, mitochondrial phosphate transporter; $\Delta$pH$_{i}$, mitochondria inner membrane pH gradient; $\Delta$Ψ, mitochondria inner membrane potential; MV$_{G}$, mitochondrial oxygen consumption; TPP$^+$, tetraphenylphosphonium cation; SNARF, seminaphthorhodafluors; ANEPPS, aminonaphthylethylpyridinium; FCCP, carbonyl cyanide $p$-trifluoromethoxyphenylhydrazone; pH$_{m}$, mitochondrial matrix pH; cyto-a, cytochrome a; cyto-c, cytochrome c absorbance; (cyto-c)$_{hv}$, oxidized cytochrome absorbance; (cyto-c)$_{hv,}$ reduced cytochrome absorbance; ED-FRAP, enzyme-dependent fluorescence recovery after photobleaching; G/M, glutamate and malate; $\Delta$G$_{H}$NADH, free energy of mitochondrial NADH; NMR, nuclear magnetic resonance; CR, creatine kinase; CrP, creatine phosphate.
heart mitochondria were used with simultaneous measurements of ATP synthetic rates, NADH, cytochrome redox potentials, $\Delta mH^+$, and $\Delta \phi$ during systematic alterations of the [P]/[I]. The results of these studies suggest that $P_i$ is remarkably well suited to regulate oxidative phosphorylation on several levels, including the generation of NADH, the flow of reducing equivalents in the cytochrome chain, and as a substrate for ATP production. As a result of these multiple sites of action, $P_i$ has the potential of providing the balanced activation of mitochondrial ATP production in the mitochondrial network controlling oxidative phosphorylation.

**EXPERIMENTAL PROCEDURES**

**Porcine Heart and Skeletal Muscle Mitochondria Isolation**—Heart mitochondria were isolated from *in situ* perfused porcine hearts as described previously (21). For comparison purposes, skeletal muscle mitochondria were prepared using the same protocol without *in situ* perfusion. The standard experimental buffer for these studies was buffer A, composed of 125.0 mM KCl; 15.0 mM NaCl; 20.0 mM K-HEPES; 1.0 mM KHCO$_3$; 1.0 mM K$_2$EDTA; 5.0 mM MgCl$_2$; 4$\mu$m tetraphenylphosphonium (TPP$^+$), at pH 7.1. The pH needed to be titrated separately for room temperature and 37 °C. Phosphate was added as the K salt pre-titrated to pH 7.1. ATP was also added as the Na salt at pH 7.1. The free [Ca$^{2+}$] was estimated as described previously (21) and was generally held at 500–600 nm with CaCl$_2$, unless specified otherwise.

**Measurements of Mitochondrial Oxygen Consumption** ($mV_o2$) —Mitochondria were prepared using the same protocol without *in situ* perfusion, and mitochondrial oxygen consumption was measured by determining the transmission of multiple wavelengths through the mixing chamber, as described previously (25). A “white” light source (Ocean Optics) was impinged on the mitochondria suspension via a fiber optic, whereas the transmitted light was directed to the same spectrophotometer used for the fluorescence measurements. Optical absorbance was calculated by taking the log of the ratio of transmitted light in the presence of buffer (Lb) and in the presence of the suspension (Ls). The spectral characteristics were determined at the isobestic frequencies of this suspension and summed.

**Mitochondrial Optical Absorption Studies**—To minimize the mitochondrial light scattering effects on the measurement of the optical absorbance properties, an integrating sphere was used with a centered-mounted cuvette (1 × 1 cm) (PerkinElmer Life Sciences model Lambda 800). Due to the configuration of this instrument, all experiments were conducted at room temperature, and no time courses were collected, because the sample had to be removed to make additions. By using this approach, all of the photons scattered by the sample were collected and analyzed. The only corrections necessary were for background absorbance and path length calibrations. This arrangement permitted the simultaneous monitoring of the entire UV and visible spectra of the mitochondria, from 310 to 700 nm. Quantitative analysis was performed by calculating the $\Delta$ of peak absorbance (i.e. sample) versus a line drawn between two isobestic reference points (i.e. reference) (26). These wavelength combinations were as follows: cytochrome c (cyto-c) sample:550 nm and cytochrome b (cyto-b) sample:563 nm, both referenced between 535 nm and 575 nm; cytochrome a (cyto-a):sample 605 nm, referenced between 575 nm and 630 nm. No attempt to quantitate the reduction levels using the $\gamma$ band was attempted, due to the overlap in the oxidized absorbance at ~414 nm, which was shared by all of the cytochromes. The $\gamma$ peaks were useful for a qualitative independent confirmation of the reduction level changes of the cytochromes, because the extinction differences between the oxidized and reduced cytochromes were roughly 10 times higher in this region. All optical absorbance data are reported as the % reduction level, as determined in Equation 4.

**Perfused Heart Optical Spectroscopy**—The resting redox state of the mitochondrial cytochromes was determined in a Langendorff perfused heart preparation, as described previously (27). Briefly, the rabbits were anesthetized by an IV injection of ketamine HCl (20 mg/kg) with acepromazine maleate (0.2 mg/kg) and were anticoagulated by an IV injection of heparin (2000 units). The hearts were arrested by an intravenous injection of KCl (4 mEq), quickly excised, and perfused at 37 °C with a modified high KCl Krebs-Henseleit solution composed of 115 mM NaCl, 25 mM KCl, 1.6 mM CaCl$_2$, 1.4 mM MgSO$_4$, 1.0 mM KH$_2$PO$_4$, 25 mM NaHCO$_3$, 5.6 glutamate, 3.0 mM l-tartaric acid, and 3.0 mM NaOH and gassed with 95% O$_2$, 5% CO$_2$. The high KCl perfusate maintained the arrested condition, which minimized the motion of the heart and reduced the cytosolic P, to the lowest values possible (27). By using a bifurcated fiber optic (Oceans Optics), a white light source (Titan Tool, FO-150) was impinged on the free left ventricle wall of the heart. The red light was collected through the other fiber of the cable and directed to a rapid scanning spectrometer (Oceans Optics, PC2000) embedded in a personal computer. Optical spectra were continuously obtained from 300 to 700 nm. After collecting a baseline control, 1 μM KCN was added to the perfusate to reduce selectively the mitochondrial cytochromes without influencing the myoglobin oxygenation (see Ref. 16). Cytochrome optical differences were calculated using the same procedures as outlined above for mitochondria.

**NADH Enzyme-dependent Fluorescence Recovery after Photobleaching (ED-FRAP)** —The NADH ED-FRAP method (29) was used to measure the capacity of the mitochondria to generate NADH by summing all
of the dehydrogenase activity using a previously described micro-photolysis system (30). NADH ED-FRAP measures the dehydrogenase activity by rapidly photolyzing NADH to NAD and observing the rate of NADH re-synthesis. NADH ED-FRAP experiments were conducted at room temperature due to the complexity of the irradiation apparatus. To facilitate their correlation with other measurements, studies were routinely conducted by using a mitochondria suspension (1.6 ml) in a sealed water-jacketed chamber at room temperature. By using a Clark-type oxygen electrode (YSI Inc.), the MVO₂ was monitored in this well mixed chamber. When a steady state was attained, a 50-μl sample was removed from the chamber, injected into the small quartz cuvette, and immediately analyzed by the NADH ED-FRAP method (30).

The NADH ED-FRAP studies were all performed in buffer A with 5 mM glutamate and malate (G/M) for carbon substrates and 500 nM free Ca²⁺. Rotenone was added to prevent any net flux or competing FADH-linked reactions in the NADH recovery process (29, 30). The NADH fluorescence was continuously monitored in the 50-μl cuvette, using a fiber optic rapid scanning spectrophotometer (Oceans Optics) and a UV excitation source. Data are reported as the initial rate of NADH regeneration from NAD after photolysis.

RESULTS

Two basic protocols were followed in this study. The first protocol was a dose-response of [Pi] on quiescent mitochondria (no net oxidative phosphorylation), performed by adding Pi in the absence of ADP. ADP was then added to evaluate the active state during net ATP synthesis. The second protocol involved adding ADP first and then performing a dose-response of [Pi] to activate ATP production. These two protocols permitted the separation of the individual effects of these key substrates for the F₁F₀-ATPase reaction.

Effects of Pi on “Quiescent” Mitochondria—The effects of the Pi on the resting [NADH], ΔΨ, and ΔPHₐ in the absence of ADP were determined. An example from this series is presented in Fig. 1A, with the data summarized in Table I. The mitochondria were initially energized with 5 mM G/M at the time indicated. The addition of G/M increased [NADH], decreased ΔPHₐ, hyperpolarized ΔΨ, and increased MVO₂, consistent with the energization of the inner membrane by the G/M-supported dehydrogenase activity. The increase in MVO₂ is likely due to an increase in the leak current, due to the hyperpolarization (31). Pi (3 mM) increased the [NADH], decreased ΔPHₐ, hyperpolarized ΔΨ, and increased MVO₂. The calculated ΔGₚₐ increased despite the reduction in ΔPHₐ due to the Pi activity (Table I).

These results are inconsistent with an activation of the F₁F₀-ATPase activity by Pi in conjunction with the residual ADP in the matrix, because that would have depolarized ΔΨ and decreased the [NADH] as well as ΔGₚₐ. In several experiments (~30%), the resting NADH levels were nearly 90% reduced after the G/M addition, resulting in much smaller, but qualitatively similar, effects of Pi on the NADH levels. This was accompanied by the very low resting respiratory rates, suggesting that these particular preparations had exceedingly low leak values resulting in the high resting NADH levels. These preparations were not significantly different from other preparations with regard to all of the measured parameters in the active (i.e. presence of ADP and Pi) condition, where the membrane resistance was dominated by the F₁F₀-ATPase activity.

The dose-response curves for Pi on resting NADH (normal-
Phosphate Regulation of Oxidative Phosphorylation

Effects of P and ADP on heart mitochondria

| G/M (control) | P (3 mM) | ADP (1.3 mM) | Active state |
|---------------|----------|--------------|-------------|
| MV0<sub>d</sub> | 30.8 ± 0.9 | 40 ± 3.4<sup>c</sup> | 24.6 ± 4.4<sup>c</sup> | 256 ± 20<sup>c</sup> |
| NADH<sup>B</sup> | 60 ± 3 | 76 ± 9<sup>c</sup> | 45 ± 7<sup>c</sup> | 29 ± 8<sup>c</sup> |
| Δψ | −172 ± 2.0 | −194 ± 6.0<sup>c</sup> | −148 ± 1.0<sup>c</sup> | −163 ± 4<sup>c</sup> |
| pHi<sub>d</sub> | 7.14 ± 0.02 | 7.13 ± 0.2<sup>c</sup> | 7.16 ± 0.05<sup>c</sup> | 7.10 ± 0.2<sup>c</sup> |
| ΔG<sub>NADH</sub> | −155 ± 2.0 | −195 ± 6.0<sup>c</sup> | −152 ± 1.9<sup>c</sup> | −163 ± 5<sup>c</sup> |
| N | 20 | 8 | 10 | 18 |

<sup>a</sup> P<sub>d</sub> added to resting mitochondria (Fig. 1).
<sup>b</sup> ADP added to resting mitochondria (Fig. 3).
<sup>c</sup> Combined data for all experiments after both ADP and P were added.
<sup>d</sup> Nanomoles O<sub>2</sub>/min/nmol cyt.
<sup>e</sup> Paired t test to control p < 0.05
<sup>f</sup> % NADH versus anoxia (100).
<sup>g</sup> Millivolts.

The effects of Pi and ADP on heart mitochondria (Table I). The order of addition of ADP or Pi had no measurable effect on the NADH concentration, due to the sensitivity of the NADH generating capacity by the F<sub>1</sub>F<sub>0</sub>-ATPase. The addition of ADP (1.3 mM), in the absence of Pi, resulted in a decrease in the [NADH], an increase of 3 mM Pi resulted in a large increase (∼10-fold) in the [NADH], whereas the [NADH] was significantly decreased.

When Pi was added after ADP (Fig. 2), another putative cytosolic signaling molecule capable of controlling multiple steps within oxidative phosphorylation (8), were evaluated under these active conditions by performing these studies in the presence and absence of added Ca<sup>2+</sup>. The addition of Ca<sup>2+</sup> (500 nM free) did not affect the calculated K<sub>p</sub> for P<sub>i</sub> for MV<sub>O</sub>, but it did increase the maximum rate of respiration by 120 ± 15% (data not shown).

The effects of P<sub>i</sub> on the Maximum Rate of ATP Synthesis—The effects of P<sub>i</sub> on mitochondrial metabolism were evaluated at the maximum rate of ATP production. Examples of these studies are presented in Figs. 1 and 3, in which both ADP and P were present. The order of addition of ADP or P had no measurable effect on the steady state processes, and the data are combined in Table I, but the approaches to this new steady state under the two conditions are revealing. The maximum rate of ATP production was estimated from the initial rate of respiration, after the addition of ADP or P to the system. This was done to avoid any effects of P or ADP consumption on the observed rate. All experiments were conducted in the absence of added ATP.

In the first series, ADP was added after P<sub>i</sub>. Under these conditions, the addition of ADP was accompanied by a large increase in respiration, associated with a depolarization of ΔΨ from its highly polarized state back to its control levels with G/M alone, whereas the [NADH] was significantly decreased.

When P<sub>i</sub> was added after ADP (Fig. 2A and Table I), the combined effects of P<sub>i</sub> on the F<sub>1</sub>F<sub>0</sub>-ATPase, NADH generation, and redox equivalent flux can be observed. With the addition of P<sub>i</sub>, the [NADH] decreased, suggesting that the rate of NADH oxidation initially exceeded the rate of production, even with the predicted activation of the NADH generating capacity by P<sub>i</sub>. However, ΔΨ hyperpolarized while ΔpH<sub>n</sub> decreased, and surprisingly, ∆G<sub>II</sub> remained essentially constant or increased slightly. The constant ∆G<sub>II</sub> is surprising, because the reducing equivalent flux through NADH had increased more than 10-fold, and ∆G<sub>NADH</sub>, the driving force for ∆G<sub>II</sub>, had been significantly reduced. These latter data suggest that P<sub>i</sub> is causing a more efficient transmission of the ∆G<sub>NADH</sub> to ∆G<sub>II</sub>.

The dose dependence plots of P<sub>i</sub> on NADH, ΔΨ, and MV<sub>O</sub> in the active state are presented in Fig. 2, B and C. Note that P<sub>i</sub> increased ΔΨ and MV<sub>O</sub> in almost an identical fashion, with K<sub>p</sub> values of 1.0 mM MV<sub>O</sub> and 0.8 mM ΔΨ obtained by simply modeling the data by using Equation 2. However, the complex effect on NADH resulted in an initial drop in the [NADH], which remained essentially constant with all subsequent additions. Modeling this as a simple exponential decay resulted in a K<sub>i</sub> of 0.08 mM.

The effects of Ca<sup>2+</sup>, another putative cytosolic signaling molecule capable of controlling multiple steps within oxidative phosphorylation (8), were evaluated under these active conditions by performing these studies in the presence and absence of added Ca<sup>2+</sup>. The addition of Ca<sup>2+</sup> (500 nM free) did not affect the calculated K<sub>p</sub> for P<sub>i</sub> for MV<sub>O</sub>, but it did increase the maximum rate of respiration by 120 ± 15% (data not shown).

Thus, the P<sub>i</sub> and Ca<sup>2+</sup> effects are additive on oxidative phosphorylation.

Effects of P<sub>i</sub> on NADH ED-FRAP—NADH ED-FRAP experiments were conducted in intact mitochondria to confirm the direct activation of the dehydrogenase activity by P<sub>i</sub>. NADH ED-FRAP permits the direct observation of the net production of NADH in intact mitochondria. These studies were conducted at room temperature (−25°C) on suspensions of heart mitochondria (2 mg/ml) in the presence of 600 mM free Ca<sup>2+</sup> and 5 mM G/M. To avoid any effect of reverse or forward electron flow in the cytochrome chain on this measurement, rotenone (40 μM) was added to the medium to stop electron transfer beyond site 1. The addition of rotenone also forced the NADH level to essentially 100%, eliminating any base-line differences in the [NADH] between the control and P<sub>i</sub> additions. P<sub>i</sub> increased the initial rate of NADH regeneration, as shown in Fig. 3A. These data are consistent with P<sub>i</sub> activating the net NADH generation capacity of the mitochondria, at the level of dehydrogenases or site 1.

Effects of P<sub>i</sub> on Uncoupled Respiration—In uncoupled mitochondria the reducing equivalent flux is independent of the F<sub>1</sub>F<sub>0</sub>-ATPase activity, and the actions of P<sub>i</sub> on reducing equivalent generation can be directly observed. The addition of 0.2 mM FCCP in the presence of 5 mM G/M and 600 mM free Ca<sup>2+</sup>, without ADP, ATP, or P<sub>i</sub>, resulted in a transient increase in MV<sub>O</sub> followed by an inhibition (Fig. 3B). The subsequent addition of 3 mM P<sub>i</sub> resulted in a large increase (∼10-fold) in the...
respiratory rate, coincident with an increase in [NADH] and the hyperpolarization of \( \frac{\Delta V}{H} \). These data, summarized in Table II, are consistent with the notion that Pi activates NADH generation.

Effects of Pi on the Mitochondrial Cytochrome Redox States—The evidence presented above suggested that Pi enhances coupling between \( \frac{\Delta G}{H} \text{NADH} \) and \( \frac{\Delta G}{H} \text{H} \). This enhanced coupling implies that a more efficient distribution of redox potential energy might be occurring down the cytochrome chain, increasing the available driving force for proton extrusion at sites II and III. The cytochrome redox state was evaluated using both the \( \frac{\Delta G}{H} \text{NADH} \) and \( \frac{\Delta G}{H} \text{H} \) absorbance bands to test this hypothesis. The absolute absorbance spectra are presented in Fig. 4 for fully oxidized (no carbon substrate, ADP, and Pi) and fully reduced (5 mM G/M at anoxia) mitochondria, and a difference spectrum is presented in Fig. 4B. The spectral assignments are as follows: cytochrome \( a \) and \( a_3 \), 605 and 444 nm; cyto-b, 563 and 430 nm; and cyto-c, 550 and 416 nm. NADH and FAD have broad absorbance bands centered at 340 and 465 nm. The absolute absorption spectrum of FAD is difficult to assess, due to the strong interference from the cytochrome absorbance.

To evaluate the effect of Pi on the cytochrome redox state, experiments were conducted in which Pi (3 mM) was added, before or after ADP (1.3 mM), to mitochondria incubated in the presence of 600 nM Ca\(^{2+}\) and 5 mM G/M. Difference spectra for ADP (0.3 mM) versus the control or 3 mM phosphate versus control are presented in Fig. 4C. The addition of Pi resulted in the oxidation of cyto-b, as seen at both 430 and 565 nm. However, cyto-c became more reduced, as seen at 416 and 550 nm. ADP additions in the absence of Pi had no significant effect on the cytochrome redox state. Quantitative redox changes are presented in Table III. Whether Pi was added alone or after the addition of ADP, cyto-b became more oxidized, and cyto-c became more reduced. These data are consistent with the notion that Pi enhances the ability of cyto-b to maintain cyto-c in the reduced state under these conditions.

Cytochrome Reduction Level in the Perfused Rabbit Heart—To determine whether a similar reduction level gradient between cyto-b and cyto-c exists in the intact heart, the cytochrome reduction level in perfused rabbit hearts was determined during KCl arrest, when the tissue Pi was below detectable limits by \(^{31}P\) NMR (<0.1 mM) (27). An example of these studies is presented in Fig. 5. The difference spectra between the KCl-arrested heart and the fully reduced state with KCN reveals no significant absorbance in the cyto-b region at 563 nm, as compared with fully oxidized versus reduced isolated heart mitochondria or homogenates made from intact rabbit heart (Fig. 5, inset). The optical difference value for cyto-b and cyto-c was calculated using the isosbestic points for the cytochromes described earlier. The ratio of the optical difference under these conditions (i.e., control versus KCN infusion) of cyto-c/cyto-b in the perfused heart was 7.3 \( \pm \) 0.8 (n = 5).
In rabbit whole heart homogenates, treated with rotenone to generate a fully oxidized cytochrome condition and KCN/succinate for creating a fully reduced condition, the optical difference ratio of cyto-c/cyto-b was only 2.5/H11006/H0.1. These data are consistent with the notion that a large gradient in reduction level exists between cyto-b and cyto-c in the arrested heart. Similar results were obtained from earlier quantitative spectral studies on intact guinea pig hearts, where little or no oxidized cyto-b was detected (32).

Effect of Pi on Mitochondrial Matrix Volume—Light scattering was used to qualitatively monitor the matrix volume, with each preparation used as its own control. Fig. 6A illustrates an experiment in this series. The addition of G/M results in an increase in absorbance (due to increased scattering) consistent with a decrease in the matrix volume. The subsequent addition of 3 mM Pi decreases the light scattering, consistent with pronounced matrix swelling, as reported previously (14). The subsequent addition of ADP (300 μM) increased the absorbance, consistent with a reduction in matrix volume. The dose dependence of the absorbance changes with Pi is presented in Fig. 6B. The Km for scattering in resting mitochondria was 4.5 mM Pi, almost an order of magnitude higher than the Pi Km for [NADH] or/H9004/H9023/under these conditions (Fig. 1B).

The lack of correlation between the mitochondrial volume and the metabolic effects of Pi is highlighted by the effect of Pi on FCCP-treated mitochondria. Fig. 6C shows the scattering and oxygen consumption effects of FCCP and Pi. Pi hyperpolarizes ΔΨ and increases the [NADH] as well as MVO2 in the presence of FCCP (Fig. 4). However, after a very brief swelling period, Pi results in a decrease in the matrix volume in the presence of FCCP, which is correlated with the increase in MVo2. These data suggest that many of the metabolic effects of Pi cannot be ascribed to matrix volume changes alone.

The significant changes in the matrix volume with Pi may interfere with ΔΨ determinations using the TPP+ distribution. To evaluate qualitatively if the Pi-induced changes in the matrix volume could influence the measurement of ΔΨ with TPP+, paired experiments were conducted with TPP+ and di-4-ANEPPS emission studies. In these studies, di-4-ANEPPS emission followed TPP+ under all conditions studied (data not shown), suggesting that the volume changes associated with Pi are not significantly influencing the determination of ΔΨ by the TPP+ distribution.

**Table II**

|                     | Control | FCCP (1 μM) | Pi (3 μM) |
|---------------------|---------|-------------|-----------|
| MVO2 (nmol/min/mmol cyto-a) | 44 ± 3.8 | 30.0 ± 5.2  | 251 ± 20   |
| NADH (%)           | 52.5 ± 7.5 | 1.0 ± 2.2   | 19 ± 5.5   |
| ΔΨ (mV)            | −148 ± 4.8 | −113 ± 6.9  | −139 ± 5.4 |

* Nanomoles of O2/min/mmol cyto-a.

* Paired t test p < 0.05.

* % NADH versus anoxia (100).
Skeletal Muscle Mitochondria—Several comparative studies were performed on skeletal muscle mitochondria to evaluate whether the effects observed with Pi were limited to heart mitochondria. Due to the lower yield of mitochondria from skeletal muscle, presumably related to the much lower concentration/gram of muscle, experiments were conducted at half the concentration of mitochondria (0.5 nmol of cyto-a/ml) and were somewhat limited in number. The matrix pH was not determined in these studies. As summarized in Table IV, the enhancement of the dehydrogenase activity in the skeletal muscle mitochondria was more pronounced than that in the heart mitochondria, based on the larger increase in $\Delta G_{\text{NADH}}$ with the addition of $P_i$ in the resting or active state. Otherwise, the overall effects of $P_i$ on the parameters measured were very similar in heart and skeletal muscle mitochondria.

**DISCUSSION**

This study demonstrates that extra-mitochondrial $P_i$ can modulate the rate of mitochondrial ATP production on several levels in vitro. Evidence is presented that $P_i$ activates the mitochondrial NADH-generating capacity and improves the distribution of energy between cyto-b and cyto-c as well as serving as the primary substrate for the $F_1F_0$-ATPase production of ATP. These effects are summarized schematically in Fig. 7. These sites of $P_i$ activation suggest the efficient balanced activation of oxidative phosphorylation by this putative cytosolic signaling molecule.

The $P_i$-induced increase in the mitochondrial dehydrogenase activity was directly demonstrated using NADH ED-FRAP in intact mitochondria. The $>70\%$ activation of the mitochondrial NADH generation capacity suggests that $P_i$ impacts several dehydrogenases simultaneously. This is supported by the net increase in the [NADH] in resting mitochondria and during uncoupled respiration in heart mitochondria. This was even more prominent in skeletal muscle mitochondria, where a net increase in $\Delta G_{\text{NADH}}$ was observed even when the addition of $P_i$ was associated with a $>5$-fold increase in NADH consumption. Previous studies demonstrated that $P_i$ can activate Krebs cycle dehydrogenases including 2-oxoglutarate dehydrogenase (19), NAD-isocitrate dehydrogenase (33), and malate dehydrogenase (34). Which, if any, of these enzymes are responsible for the $P_i$ effects observed in this study is unknown. Different carbon substrates were used to probe different NADH-generating.

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**TABLE III**

|                  | Cyto-b (\% reduced) | Cyto-c (\% reduced) |
|------------------|---------------------|---------------------|
| Control (no ADP)| 46.7 ± 1.5          | 9.2 ± 0.7           |
| $P_i$ (3 mM)     | 39.0 ± 1.7          | 10.5 ± 0.8          |
| Control (1 mM ADP)| 53 ± 2.6          | 8.4 ± 0.8           |
| $P_i$ (3 mM)     | 38 ± 1.6            | 17.1 ± 1.2          |

* Paired t test versus control $p < 0.05$. 

**Fig. 4.** Optical spectra of isolated heart mitochondria, using an optical integrating sphere. A, absolute spectra of mitochondria (1 nmol cyto-a/ml) in the fully oxidized (no carbon substrate, ADP, and $P_i$) and fully reduced (G/M at anoxia) forms. B, difference spectrum (reduced-oxidized). C, serial difference spectra of additions to the heart mitochondria suspension, starting with ADP and followed by $P_i$. 

**Skeletal Muscle Mitochondria**—Several comparative studies were performed on skeletal muscle mitochondria to evaluate whether the effects observed with $P_i$ were limited to heart mitochondria. Due to the lower yield of mitochondria from skeletal muscle, presumably related to the much lower concentration/gram of muscle, experiments were conducted at half the concentration of mitochondria (0.5 nmol of cyto-a/ml) and were somewhat limited in number. The matrix pH was not determined in these studies. As summarized in Table IV, the enhancement of the dehydrogenase activity in the skeletal muscle mitochondria was more pronounced than that in the heart mitochondria, based on the larger increase in $\Delta G_{\text{NADH}}$ with the addition of $P_i$ in the resting or active state. Otherwise, the overall effects of $P_i$ on the parameters measured were very similar in heart and skeletal muscle mitochondria.
pathways. \( P_i \)-activated NADH generation with pyruvate or carnitine-palmitate (not shown) was very similar to glutamate/malate, despite the fact that these substrates are oxidized through different pathways. \( P_i \) activation apparently works globally on the dehydrogenase activity, consistent with \( P_i \) activation of numerous dehydrogenases \textit{in vitro}. However, specific enzymatic confirmation of these processes in extracts or with other substrate/inhibitor regimes will be required to refine further the dehydrogenase activation process. It is also interesting to note that ADP, the other substrate for the \( F_1F_0 \)-ATPase and a putative cytosolic signaling molecule, did not demonstrate any activation of the dehydrogenase activity, in either the steady state measurements (Fig. 2) or the NADH ED-FRAP measurements (30). Thus, despite the fact that ADP has been reported to activate several dehydrogenase reactions \textit{in vitro}, no evidence for this interaction was observed.

One of the unique findings in the current study was the discrepancy between \( \Delta G_{\text{NADH}} \) and \( \Delta G_H \) with the initiation of oxidative phosphorylation with \( P_i \) (Fig. 2 and Table I). The maintenance of \( \Delta G_H \) as \( \Delta G_{\text{NADH}} \) declines during this transition suggests that \( P_i \) facilitates the coupling between \( \Delta G_H \) and \( \Delta G_{\text{NADH}} \). A spectroscopic investigation of the reduction state of the cytochrome chain revealed that this increased coupling was associated with a crossover between cyto-b and cyto-c, \textit{i.e.} cyto-b becomes more oxidized as cyto-c becomes reduced. One would predict that this increase in cyto-c reduction level enhances the available potential energy in the last two proton ejection sites of the cytochrome chain, thereby increasing the ability to generate \( \Delta \Psi \). The underlying mechanism of the coupling enhancement between cyto-b and cyto-c is unknown. Possible mechanisms include the mobility of cyto-c in the matrix membrane and the redox coupling of cyto-c1 and cyto-b as well as cyto-c. To our knowledge, no studies of the effects of \( P_i \) on the redox coupling of cyto-c and cyto-b \textit{in vitro} have been performed.

One of the consequences of the electron flow control between cyto-b and cyto-c is that cyto-c will remain relatively oxidized, whereas cyto-b will be highly reduced under inactive, arrested conditions in the heart, where the free \( P_i \) is extremely low. This was confirmed in the arrested perfused rabbit heart, where the cyto-b was almost fully reduced whereas the cyto-c was highly oxidized (Fig. 5). Similar results for the guinea pig heart were obtained by Hoffmann \textit{et al.} (32) where no oxidized cyto-b could be detected. We also confirmed this redox poise between cyto-b and cyto-c in isolated rabbit heart myocytes, placed in the integrating sphere system, using an uncoupler (FCCP) and KCN to generate oxidized and reduced reference points (data not shown). These intact tissue studies are consistent with an incomplete equilibrium between cyto-b and cyto-c in resting tissues with low \( P_i \). It is interesting to consider the consequences of the inhibition of electron flow between cyto-b and cyto-c. Site III might be an important site of reactive oxygen species generation in the heart (35). We speculate that by keeping cyto-c as oxidized as possible, to just meet the ATP synthetic needs of the tissue, the generation of free radicals in the mitochondria may be minimized. These data suggest that the flux of reducing equivalents between cyto-b and cyto-c is highly regulated, and this step is under the control of \( [P_i] \) and likely other factors.

Based on the steady state kinetics of the \( P_i \) effects on mitochondrial metabolism, what role would \( P_i \) predictably play in the regulation of oxidative phosphorylation in the intact cell? \( P_i \) has been considered for some time to play a role in regulatory cytosolic feedback, by balancing ATP hydrolysis with oxidative phosphorylation (1, 2). However, the role of \( P_i \) in the regulation of oxidative phosphorylation \textit{in vivo} has usually been consid-
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considered to be small, due to the relatively high affinity for the metabolic effects as compared with the tissue concentrations. The resting concentration of Pi in heart and skeletal muscle has been a controversial issue for some time. Physical or chemical extraction of tissue yields very high Pi concentrations, on the order of several mM (36). However, several investigators using 31P NMR approaches have shown that most of this extracted Pi is immobile and not chemically active (36, 37). In perfused hearts, the Pi concentration has ranged from being essentially undetectable in arrested or pyruvate-perfused hearts (27, 36) to 1.3 to 1.8 mM with glucose (36). By using quantitative 31P NMR spectroscopy in the canine heart in vivo, Katz et al. (38) determined the Pi concentration to be $800 \mu M$ at rest, which is close to the $K_p$ value determined in this study for many of the observed metabolic effects. Despite the fact that the [Pi] is close to the $K_p$, the [Pi] is very stable in the heart during physiological changes in workload (39, 40). However, at near-maximum workloads (39, 41), with pharmacological stimulation (42) or during partial ischemia or hypoxia, increases in [Pi] have been observed in the heart by several groups. Thus, under these conditions, changes in the [Pi] might contribute to the regulation of cardiac oxidative phosphorylation. In the post-ischemic condition, the high [Pi] might contribute to free radical generation (35) due to the enhanced reducing equivalent delivery to cyto-c.

In human skeletal muscle, the resting [Pi] is higher than that in the heart and approaches 4 mM, but it is much more labile to changes in the workload, due to the breakdown of creatine phosphate (CrP) (43) (further discussed below). However, based on the steady state $K_p$ ($500 \mu M$) determined for heart mitochondria, it is likely that the effects of Pi might already be maximized in skeletal muscle, even at rest. It has been proposed that most of the previous measurements of metabolites using NMR or classical chemical techniques might not accurately reflect the metabolite pools in the vicinity of the mitochondria or, more importantly, in the inner membrane space of the mitochondria (for example see Refs. 44 and 45). This is especially a concern because $\Delta \mu_{Pi}$, the driving force for Pi, is unknown in the intact muscle. Although these hypotheses lack ample direct experimental evidence, it is possible that the cytosolic compartmentation of Pi could complicate the interpretations of these bulk biochemical assays.

With regard to the mechanisms of Pi action on these different sites in oxidative phosphorylation, very few are understood, but several conclusions can be made. First, the net effects of Pi are additive with Ca$^{2+}$, so it is unlikely that the modification of

**TABLE IV**

Effect of Pi on skeletal muscle mitochondria

| Units and conditions are same as in Table I. |
|---------------------------------------------|
| G/M (control) | Pi (3 mM) | ADP (1.3 mM) | Active state |
| MV$_{3D}$ | 27 ± 2 (6) | 39 ± 3* (4) | 30 ± 4 (2) | 186 ± 10* (4) |
| NADH | 54 ± 3 (5) | 84 ± 4* (4) | 46 (1) | 69 ± 1 (3) |
| $\Delta \phi$ | $-148 ± 2 (5)$ | $-183 ± 0.3* (3)$ | $-143 ± 2 (2)$ | $-162 ± 8* (4)$ |

Fig. 6. Light scattering and respiration in isolated heart mitochondria. The absorbance was determined at the cytochrome isosbestic point at 535 nm. A, control mitochondria. B, dose-response curve of Pi on light scattering in resting mitochondria. C, effect of Pi in the presence of FCCP.
matrix Ca\(^{2+}\) by P\(_1\) chelation is occurring. This additive behavior also suggests that Ca\(^{2+}\) and P\(_1\) are acting on different sites. The difficulty in determining the mechanism of P\(_1\) action is related to the numerous aspects of the matrix milieu that are affected by this agent. For example, P\(_1\) alters both the matrix pH and volume, which could indirectly affect several metabolic reactions. The matrix membrane pH gradient was small (<0.1 pH unit) in this preparation, as compared with those of previous studies at room temperature in simpler media (24). The low ΔpH\(_m\) was presumably a consequence of the higher temperature (37 °C) and the complex ionic incubation medium. Specifically, we found that just the addition of Ca\(^{2+}\) reduced ΔpH\(_m\) ~0.05 (not shown). As a result of the small ΔpH\(_m\), the effects of P\(_1\) on pH\(_m\) were very limited, only changing <0.05 in the steady state. Most enzymatic processes require much larger changes in the pH to change the kinetics by the magnitude (2–3-fold) observed in this study. We attempted to clamp the pH\(_m\) using potassium acetate (2–10 mM) and several other organic ions. However, we found that acetate nearly abolished the ΔpH\(_m\) in a similar fashion as P\(_1\) but caused a marked inhibition of both the NADH generating capacity and the maximum ATP production rates under control conditions. Because these are the opposite effects of P\(_1\), we discontinued these studies and assumed that these organic ions were exerting other nonspecific effects. We believe that the small magnitude of the changes in pH\(_m\) with P\(_1\) makes it unlikely that it is primarily responsible for the observed metabolic changes. P\(_1\) also results in marked increases in the matrix volume, which could have numerous metabolic consequences (15). However, the P\(_1\) dose response for the matrix volume was nearly 5 times that required for the observed metabolic effects. One of the most significant dissociation of P\(_1\)-induced swelling from its metabolic actions was the effect of P\(_1\) in uncoupled mitochondria. P\(_1\) addition after uncoupling revealed most of the metabolic effects, such as the increase in ΔΨ and ΔG\(_{\text{NADH}}\), whereas P\(_1\) decreased volume under these conditions. Thus, it is unlikely that the volume alone is responsible for the metabolic effects of P\(_1\). One clear mechanism is that P\(_1\) activates the F\(_1\)F\(_0\)-ATPase by providing a substrate for ADP phosphorylation. To our knowledge, the allosteric interactions of P\(_1\) on the F\(_1\)F\(_0\)-ATPase have not been evaluated. Based on these observations, we believe the most reasonable hypothesis is that the matrix P\(_1\) is directly modulating the dehydrogenase activity, via some of the mechanisms already described \textit{in vitro}, as well as the redox coupling of cyto-b and cyto-c. Clearly, further investigations will be needed to determine whether this general hypothesis on the action of P\(_1\) is correct.

In considering the role of P\(_1\) in regulating oxidative phosphorylation in muscle cells, it is important to consider the role of the creatine kinase (CK) reaction in the generation of P\(_1\). With increases in ADP and P\(_1\), due to work-related ATP hydrolysis, the increases in P\(_1\) will be amplified through the CK equilibrium by effectively converting CrP into P\(_1\) (46, 47). Due to the CK equilibrium constant (1.66 × 10\(^{−9}\)), μM changes in [ADP] are reflected in ~mM decreases in [CrP] and ~mM increases in [P\(_1\)]. This is one of the reasons that \textsuperscript{31}P NMR of CrP and P\(_1\) is so useful in muscle tissues (46). Simulating a resting heart with a [P\(_1\)] of 800 μM and [ADP] of 50 μM, it can be shown that the increase in P\(_1\) is almost 150-fold that of ADP, on a mole to mole basis, during μM changes in ADP. This amplification of cytosolic [P\(_1\)] in response to [ADP] is significantly greater than the ratio of the apparent affinity constants of ADP and P\(_1\) for activating oxidative phosphorylation (K\(_{\text{mP}}\)/K\(_{\text{mADP}}\) = 800/30 μM = 27). By using these resting concentrations and K\(_{\text{mP}}\), the relative kinetic driving force for oxidative phosphorylation (i.e. [S]/K\(_{\text{mS}}\) for each substrate) can be estimated. Under these conditions, the P\(_1\) kinetic driving force rises much more quickly than ADP, during μM increases in [ADP], due to the interplay of the CK reaction. These simulations are consistent with the notion that the kinetic driving force for cardiac oxidative phosphorylation is dominated by P\(_1\), during increases in the cytosolic [ADP] through the CK reaction. If the affinity for ADP is lower in intact cells, as suggested by Saks et al. (44), then the P\(_1\) would become even more dominate. Because the CK equilibrium reaction predictably results in increasing the importance of P\(_1\), the removal of the CK should compromise the metabolic response of the heart near peak workloads. Consistent with this notion is the observation that in CK knockout mice, the skeletal and cardiac muscles do not generate the same level of P\(_1\) as in the controls, and they do not perform well while approaching maximum workloads (48). Thus, the inability of CK knockout mice to reach maximum performance may be related to the level of cytosolic P\(_1\) generated and not due to only the specific enzymology or localization of CK.

In summary, P\(_1\) was shown to activate oxidative phosphorylation at three levels: the generation of NADH, the distribution of free energy throughout the cytochrome chain, and as a substrate for ADP phosphorylation at the F\(_1\)F\(_0\)-ATPase. This balanced activation of oxidative phosphorylation results in P\(_1\) being capable of increasing the generation of ATP, without large
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swings in the concentrations or free energies of the metabolic intermediates involved in this process. These actions of Pi, working together with other cytosolic signaling networks, might help to explain the metabolic homeostasis observed in intact hearts during physiological increases in workload. Finally, the relative kinetics of the two major substrates and the putative cytosolic signaling molecules for the control of ATP production, Pi and ADP, in the presence of CK suggest that the kinetic driving force for Pi is greater than that of ADP in the intact heart. The mechanism of Pi action on these different sites is unresolved. The global effects on pH, or matrix volume do not seem to explain these phenomena. Elucidating the specific mechanisms of Pi action on the dehydrogenases and the cytchrome reducing equivalent transfer will require more studies in the future.

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