THE CAUSE OF SUPERSTOICHIOMETRIC Ca^{2+} UPTAKE AND H^+ EJECTION IN L1210 MOUSE ASCITES TUMOR MITOCHONDRIA

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Summary - Mitochondria from L1210 mouse leukemia cells show superstoichiometric $\rightarrow H^+/2e^-$ ejection and $\rightarrow Ca^{2+}/2e^-$ uptake ratios in the absence of phosphate. Kinetic analysis of Ca^{2+}-induced respiratory jumps shows a very rapid, early burst of H^+ ejection and Ca^{2+} uptake dependent on energy-coupled electron transport but with extraordinarily high stoichiometry ($\rightarrow H^+/2e^- > 20$; $\rightarrow Ca^{2+}/2e^- > 10$), followed by a slower phase with normal stoichiometry ($\rightarrow H^+/2e^- = 4\text{-}5$; $\rightarrow Ca^{2+}/2e^- = 4.0$). The early burst of H^+ ejection, which has a higher K_M for Ca^{2+} than respiratory stimulation, accounts for the superstoichiometry phenomenon; it requires energization by state 4 respiration.

It has been widely observed that 2.0 Ca^{2+} ions are accumulated from the medium by isolated animal tissue mitochondria per pair of electrons passing each energy-conserving site of the respiratory chain (i.e. $\rightarrow Ca^{2+}/2e^- = 2.0$), when phosphate is present (reviews: 1-3). However, when permeant anions are absent and the pH and salt concentration of the medium are relatively high, the observed $\rightarrow Ca^{2+}/2e^-$ ratio is much higher, values exceeding 6.0 having been observed (4-9). Such "superstoichiometric" accumulation of Ca^{2+} has been shown to depend on electron transport and to be accompanied by superstoichiometric ejection of H^+ (4, 5). The cause and mechanism of the superstoichiometry of Ca^{2+} uptake and H^+ ejection under these conditions have remained obscure (cf. 6, 9), but are of obvious importance in connection with the true value for the stoichiometry of H^+ ejection during mitochondrial and photosynthetic electron transport.

Recently we reported that mitochondria isolated from the ascites form of L1210 mouse leukemia cells show pronounced superstoichiometry of H^+ ejection and Ca^{2+} binding in a "normal" medium of 120 mM KCl and pH 7.2 (10). In these mitochondria the rates of Ca^{2+}-induced H^+ ejection and Ca^{2+}-induced respiratory stimulation do not have identical dependence on Ca^{2+} concentration; respiratory stimulation was found to have a K_M of about 8 μM Ca^{2+} and H^+ ejection a K_M of about 120 μM Ca^{2+} (10). These widely different K_M values indicated that stimulation of oxygen consumption and ejection of H^+ are not stoichiometrically coupled under these conditions.

This communication reports that the superstoichiometry in mitochondria

from L1210 mouse leukemia cells is caused by a hitherto unrecognized, very rapid, energy-dependent binding of a large amount of Ca^{2+} to the mitochondria in exchange for H^+, in a process that is dependent upon electron flow but is not stoichiometric with it. This early, rapid burst of Ca^{2+} binding and H^+ ejection, when superimposed on the much slower accumulation of Ca^{2+} and ejection of H^+ that is stoichiometric with the stimulated oxygen consumption, is responsible for the superstoichiometric $\rightarrow Ca^{2+}/2e^-$ and $\rightarrow H^+/2e^-$ ratios observed in these mitochondria in the absence of phosphate.

EXPERIMENTAL DETAILS

The traces of H^+ and of oxygen concentration, sensed by a Clark or a vibrating platinum electrode, were taken from the same reaction vessel and recorded on a dual-channel Sargent SRL recorder. The instrument responses were calibrated and corrected for the lag of the oxygen electrode which in any case was not a critical factor in the phenomena reported. The temperature was 25° in all experiments. Uptake of $^{45}Ca^{2+}$ was measured in Millipore filtrates of the medium, using a fast sampling method. The L1210 cells were cultured and the mitochondria isolated as described earlier (10, 11).

RESULTS

Since the rates of H^+ ejection and respiratory stimulation were found to have quite different dependence on the concentration of Ca^{2+} (8), with maximum H^+ ejection requiring higher Ca^{2+} concentrations, we have examined the effect of pulsed additions of Ca^{2+} at higher concentrations than previously used (8) on the time course of oxygen consumption, H^+ ejection, and Ca^{2+} uptake in L1210 mitochondria. Figure 1 (upper left) shows traces from a typical experiment in which 160 ng-atoms Ca^{2+} were added per mg mitochondrial protein in a medium of 120 mM KCl, 3.0 mM HEPES buffer pH 7.2, and 2.0 mM succinate as substrate; neither phosphate nor ADP were added. The overall H^+/O ratio was 10.0 or $\rightarrow H^+/2e^- = 5.0$ per site, clearly superstoichiometric. However, comparison of the oxygen and H^+ traces shows that a fast and large ejection of H^+ took place immediately after addition of Ca^{2+}, most of it before the onset of the increase in rate of oxygen uptake. This lag is not instrumental in origin. In order to show more clearly that H^+ ejection and oxygen consumption are not in phase, the ratio Δng-atoms H^+ ejected/Δng-atoms extra oxygen consumed for each 6 sec interval after addition of Ca^{2+} was plotted against elapsed time for the entire period of the Ca^{2+}-induced jump in oxygen consumption (Figure 1, lower left). It is seen that the $\Delta H^+/\Delta O$ ratio was extremely high at the very beginning of the respiratory jump; in fact, values exceeding 100 have been observed. The $\Delta H^+/\Delta O$ ratio

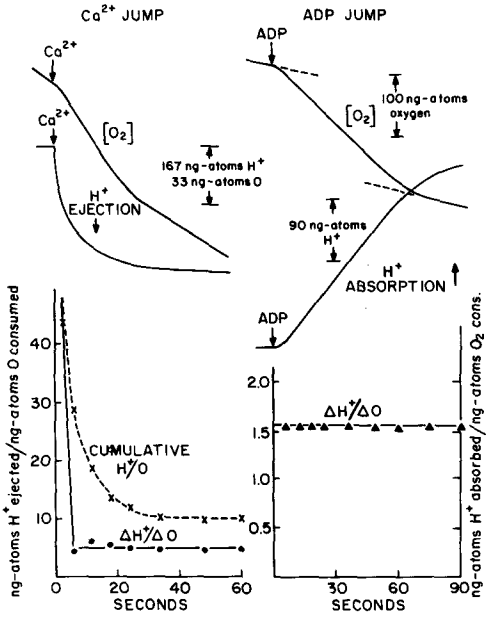

Figure 1 (Left) Early burst of superstoichiometric H^+ ejection during a Ca^{2+}-induced respiratory jump. The basic medium was 120 mM KCl, 3.0 mM HEPES buffer pH 7.2, 2.0 mM potassium succinate, 4 μM rotenone (to block endogenous respiration) and L1210 tumor mitochondria (0.96 mg protein per ml) in a total volume of 2.0 ml. At the time indicated by the arrow, 160 ng-atoms Ca^{2+} per mg protein was added and the oxygen consumption and H^+ ejection recorded. From these traces the $\Delta H^+/\Delta O$ ratios for successive 6-sec intervals were extrapolated and plotted (left, below) against elapsed time in the jump. Also shown (dotted line) is the cumulative H^+/O ratio.

(Right) The time course of H^+ absorption and oxygen consumption during oxidative phosphorylation of ADP. The medium was identical with that used in the Ca^{2+} jump but was supplemented with 0.5 mM phosphate. Instead of Ca^{2+} a total of 360 nmoles of ADP was added. A plot of the ratio ΔH^+ absorbed/ΔO consumed for successive 6-sec intervals is shown below.

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then rapidly declined over the next 6 sec and for the remainder of the jump it has a value of less than 5.0, equivalent to less than 2.5 H^+ per energy-conserving site activated, in agreement with the $\rightarrow H^+/2e^-$ ratio of ~2.0 usually observed during stoichiometric respiration-coupled transport of Ca^{2+} (1-3). Subtraction of the first rapid burst of H^+ ejection from the total H^+ ejected over the course of the jump therefore yields the usual stoichiometric $\rightarrow H^+/2e^-$ ratio of ~2.0. The experiment in Figure 1 (left) therefore shows that the early burst of Ca^{2+}-induced H^+ ejection, when superimposed on the normal H^+ ejection stoichiometric with electron transport, is responsible for the superstoichiometric H^+/O ratio during Ca^{2+}-induced respiratory jumps in the absence of phosphate.

Control experiments (Figure 1, upper right) in which ADP instead of Ca^{2+} was added to an identical system containing 2.0 mM succinate as substrate, but with 0.5 mM phosphate added, yielded stimulation of oxygen consumption (state 3) accompanied by stoichiometric absorption of H^+ from the medium, which continued until the added ADP was exhausted. In this case the ratio Δng-atoms H^+ absorbed / Δng-atoms oxygen consumed for each 6 sec interval (Figure 1, lower right) was found to be nearly constant throughout the jump with a value of about 0.86, in close agreement with the expected ratio (12), showing that the superstoichiometric burst of H^+ ejection on adding Ca^{2+} (Figure 1, left) is not an instrumental effect.

Figure 2 shows the rate of Ca^{2+} uptake measured at short intervals in an

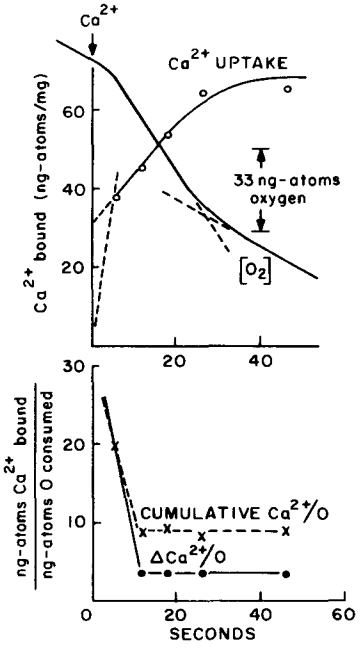

Figure 2 Early burst of superstoichiometric Ca^{2+} binding by L1210 tumor mitochondria. The test system was exactly as described in Figure 1, left. The Ca^{2+} added was labeled with $^{45}Ca^{2+}$ and samples taken at the time intervals shown. The lower plot shows the $\Delta Ca^{2+}/\Delta O$ ratio for successive short intervals during the jump, as well as the cumulative Ca^{2+}/O ratio.

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experiment duplicating that in Figure 1. Nearly half of the total Ca^{2+} accumulation occurred within the first 6 sec, followed by a slower rate of uptake. The ratio Δng-atoms Ca^{2+} bound / Δng-atoms oxygen consumed was approximately 20 in the first 6-sec interval, equivalent to a $\rightarrow Ca^{2+}/2e^-$ ratio of 10,

much larger than the normal value of 2.0. After the first rapid binding of Ca^{2+}, the $\Delta Ca^{2+}/\Delta O$ ratio in the succeeding intervals was about 3.8 or 1.9 per site, in agreement with the normal value of 2.0 Ca^{2+} per site observed when a permeant anion is present (5). In the rapid phase the ratio H^+ ejected/ Ca^{2+} bound was about 1.4 to 1.6, but declined to about 0.9 - 1.1 in the slower stoichiometric phase. Thus the experiments in Figures 1 and 2 show that in the absence of phosphate, addition of Ca^{2+} to the L1210 tumor mitochondria causes a rapid burst of H^+ ejection and simultaneous rapid binding of Ca^{2+}, thus yielding very high H^+/O and Ca^{2+}/O ratios, followed by a slower phase of Ca^{2+} uptake and H^+ ejection proportional to and presumably stoichiometric with the stimulated electron flow. It is the early, rapid burst of Ca^{2+} binding and H^+ ejection that is responsible for the superstoichiometric $\rightarrow Ca^{2+}/2e^-$ and $\rightarrow H^+/2e^-$ ratios.

The early, rapid phase of H^+ ejection can be most sharply delineated if the substrate concentration is lowered to slow down the respiratory rate. Addition of Ca^{2+} then yields a biphasic H^+ ejection trace (Figure 3), with a

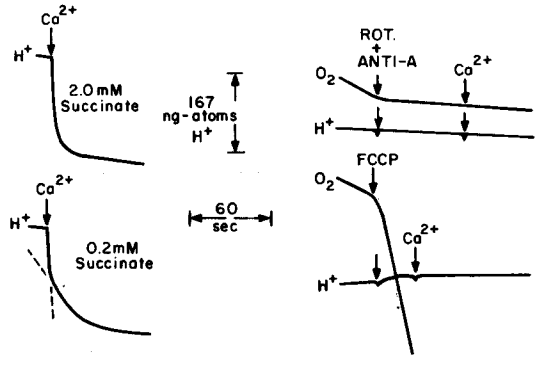

Figure 3 (Left) Biphasic H^+ ejection during Ca^{2+}-induced respiratory jump. The test system contained 120 mM KCl, 3.0 mM HEPES pH 7.2, potassium succinate as shown, and 1.25 mg mitochondrial protein per ml in a total volume of 2.0 ml. Ca^{2+} (160 ng-atoms per mg protein) was added at point shown.

(Right) Effect of inhibitors. Details as above; succinate was added at 2.0 mM, FCCP at 0.5 μM, antimycin A at 0.2 μM and rotenone at 4.0 μM.

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distinct point of inflection. The rate of H^+ ejection in the early rapid phase was at least 1600 ng-atoms H^+ per min per mg protein and the rate of Ca^{2+} binding in excess of 1200 ng-atoms Ca^{2+} per min per mg protein. The maximum amount of H^+ ejected in the early burst has been extrapolated to be about 60 ng-atoms H^+ per mg protein, corresponding to more than 40 ng-atoms Ca^{2+} per mg protein.

Other experiments in Figure 3 show that both the fast and slow phases of H^+ ejection and Ca^{2+} uptake are completely prevented by the uncoupling agent FCCP or by antimycin A + rotenone, demonstrating that the superstoichiometric portion of the Ca^{2+} uptake and H^+ ejection is not merely the result of non-specific, respiration-independent binding of Ca^{2+} to the mitochondrial membrane, such as that described in earlier studies (13). In any case, it has been shown earlier that respiration-independent Ca^{2+} binding by rat liver mitochondria is not accompanied by H^+ ejection (13, 14). The superstoichiometry phenomenon therefore appears to require an energized state of the mitochondria produced by coupled electron flow. The early burst of superstoichiometric Ca^{2+} uptake and H^+ ejection has been observed not only with succinate, but also with other respiratory substrates such as pyruvate + malate, glutamate, malate, and endogenous substrates.

When mitochondria are pretreated with rotenone to block endogenous respiration and Ca^{2+} added either before or simultaneous with the succinate there is no superstoichiometric H^+ ejection or Ca^{2+} uptake, only the slower stoichiometric type of H^+ ejection and Ca^{2+} uptake. However, when succinate is added first and Ca^{2+} 60 sec later, the usual biphasic H^+ ejection curve characteristic of the superstoichiometry phenomenon was observed. These observations show that a period of preceding state 4 electron transport is required for superstoichiometric H^+ ejection and Ca^{2+} uptake, presumably to energize the mitochondrial binding sites or to charge them with protons, before rapid exchange of Ca^{2+} from the medium with the bound H^+ can take place.

DISCUSSION

The observations described here clearly provide an explanation for the long-obscure phenomena of superstoichiometry of Ca^{2+} uptake and H^+ ejection occurring in mitochondria in the absence of permeant anions. Presumably the L1210 mitochondria contain specific binding sites that are normally occupied by protons when the mitochondria are respiring in state 4. When a pulse of Ca^{2+} is added to the state 4 mitochondria, large amounts of Ca^{2+}, up to 40 ng-atoms per mg mitochondrial protein, are very rapidly bound to the mitochondria with displacement of nearly an equivalent number of H^+ ions; the Ca^{2+} binding in this early, rapid phase bears no stoichiometric relationship with electron flow but is evidently dependent on an energized state of the mitochondria. These Ca^{2+} binding sites, which have a higher K_M for Ca^{2+} than the process by which electron transport is stimulated by Ca^{2+} (10), are functional or protonated only following a finite period of coupled electron transport in state 4. It appears possible that the low-affinity membrane

binding sites for Ca^{2+} earlier observed in respiration-inhibited mitochondria, which do not release H^+ on binding Ca^{2+} (13), may become protonated during state 4 respiration and can then release H^+ in a rapid exchange for Ca^{2+} so long as they are in a respiration-energized state.

Experiments are under way to determine whether superstoichiometry occurs under similar circumstances in mitochondria from non-malignant tissues, the location of the superstoichiometric Ca^{2+} binding sites in the mitochondria, the mechanism by which phosphate suppresses superstoichiometric Ca^{2+} uptake, and the possible biological role of the rapid, large superstoichiometric Ca^{2+} binding in the regulation of Ca^{2+}-dependent processes in cells.

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