Proton Stoichiometry of Electron Transport in Rodent Tumor Mitoplasts

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ABSTRACT

The mechanistic vectorial H+/O translocation ratios characteristic of energy-conserving sites 2 + 3 and site 3 of the respiratory chain of two tumor cell lines were determined using succinate and ferrocytochrome c, respectively, as electron donors. The measurements were carried out on mitoplasts in order to allow ferrocytochrome c free access to its binding site on the inner mitochondrial membrane. The tumor cell lines used were Ehrlich ascites tumor and the AS30-D ascites tumor. K+ was used as charge-compensating cation in the presence of valinomycin. The O2 uptake rate measurements were made with a fast-responding membraneless electrode whose response time was closely matched with that of a pH electrode. The rates of O2 uptake and H+ ejection during the apparent zero-order rate phase of respiration, analyzed by computer, were extrapolated to zero time. The observed H+/O ratios for succinate oxidation in both tumors exceeded 7 and approached 8 and the H+/O ratios for the cytochrome oxidase reaction closely approached 4.0, in agreement with data on normal mitochondria. However, the rates of H+ back decay in the tumor mitochondria are relatively high and may influence the net efficiency of oxidative phosphorylation under intracellular conditions.

INTRODUCTION

Recent progress in examination of genetic changes that may be causative of malignant transformation has focused renewed attention on characteristic phenotypic alterations in the metabolic and transport pathways of cancer cells and their regulation. Among recent advances are the observation that the terminal enzymes of glycolysis are phosphorylated by tyrosyl residues, in response to the src gene (1), the report that cytosolic malate dehydrogenase is phosphorylated under similar circumstances (2), and recent observations on the efficiency of the plasma membrane Na+-K+-ATPase (3). Changes in the pathway and regulation of glycolysis and respiration, as a consequence of transformation, may thus be fundamental in the bioenergetics of tumor cells.

The general belief in the field of bioenergetics is that progress in understanding the molecular mechanism of mitochondrial energy transduction cannot be made without first solving the still controversial question of the H+/O ratio. Until recently, the author of the chemiosmotic hypothesis postulated that the energy transduction cannot be made without first solving the still controversial question of the H+/O ratio. Until recently, the capacity of the matched O2 and pH electrodes was validated against reactions of known H+/O stoichiometry (11, 21). In order to obtain the H+/O flow ratio at approximate steady-state condition of electron flow, the observed rate data were tested against different rate laws by computer and then extrapolated to zero time. The experiments were carried out in mitoplasts, making possible use of ferrocytochrome c as electron donor, rather than the often used nonbiological reductants N,N,N’,N’-tetramethyl-p-phenylenediamine or ferricyanide. Because there is complete agreement that the H+/O ratio for site 2 alone is 4, the data obtained for succinate oxidation (sites 2 + 3) provide an important check, obtained with a normal biological substrate, on the value for site 3 obtained with ferrocytochrome c as electron donor.

MATERIALS AND METHODS

Harvesting of Tumor Cells. Two different lines of ascites tumor cells were used: AS30-D rat ascites hepatoma and Ehrlich ascites tumor cells. The tumor cells were harvested 7–9 days after i.p. inoculation of ascites fluid from donor tumor-bearing animals. Inocula of 1.0–2.0 ml of the undiluted AS30-D and of 0.15–0.2 ml of the diluted Ehrlich ascites fluids were used to maintain these tumor lines. A dilution of Ehrlich ascites fluid containing approximately 105 cells/ml in 137 mM NaCl, 2.6 mM KCl, 8 mM NaH2PO4, and 1.5 mM KH2PO4, pH 7.5 was prepared for this purpose. The AS30-D hepatoma cells were carried in 100–125-g female Sprague-Dawley rats (Holtzman, Madison, WI) and the Ehrlich ascites cells were carried in 25–30-g young adult male Swiss albino mice (Hilltop, Scottsdale, PA). All animals were fed standard laboratory chow (Agway Prolab, New Hope, PA) and tap water ad libitum.

Preparation of Mitochondria and Mitoplasts. AS30-D ascitic fluid from 4–6 rats and Ehrlich ascitic fluid from 15–20 mice were used to isolate mitochondria essentially as described by Moreadith and Fiskum (22). Mitochondria were treated with digitonin to obtain mitoplasts by the method described by Pedersen et al. (23) for liver mitoplasts. Mitoplasts that had respiratory control ratios of 4–5 with fluorocarbonyl-cyanidephenyldihydrazone present and succinate at substrate were used. Mitochondrial and mitoplast protein concentrations were determined by a modified biuret reaction (24) standardized with bovine serum albumin. Cell numbers were determined with a Howard modified counting chamber.

Determination of O2 Consumption and H+ Ejection. The reactions were carried out in a thermostatted closed cell (1.7 ml) with no gas phase (25). The O2 electrode was inserted into the side of the cell and the glass electrode into the threaded Teflon stopper. A narrow port in the stopper allowed insertion of the needle of a Hamilton syringe for injection of small volumes of reagents. The cell contents were stirred at 2000 rpm with a Teflon-coated magnetic bar driven by a motor underneath the cell. The positioning of the electrodes and the port for
cytochrome c, and from 2.5-3.2 mg protein. Other additions were 2 ¡HA points tested from the end of the dead time (~0.8 s) to over 90% of the nmol antimycin A/mg. The O2 content of the medium was reduced to rotenone-50 ng valinomycin/mg-40 nmol W-ethylmaleimide/mg-O^fonic acid, pH 7.05, and either 2.0 mM succinate or 88 ^M ferrocytochrome c, which corresponds to a simple exponential decline in rate, maintained exactly up to about 10% of air saturation at 10°C by bubbling argon before the cell was closed. The calibration of H* ejection was determined by backtitration with known amounts of 0.1 m HCl. The solubility of oxygen in the air-saturated test medium used was determined to be 632 nmol of oxygen/ml at 10°C (27). Ferricytochrome c (Type III; Sigma) was enzymatically reduced under anaerobic conditions with frozen and thawed Ehrlich mitoplasts and potassium succinate as previously desribed (12). Rates of H* ejection by AS30-D mitoplasts were measured. The test system (10°C) contained rotenone to prevent electron flow from site 1, valinomycin to allow K+ to serve as charge-compensating permeant cation, and N-ethylmaleimide to prevent H+ reuptake by symport with P¡ (10). With all other components present in the anaerobic medium the reaction was initiated by rapid injection of a precisely known amount of O2. Following a “dead period” owing to mixing and electrode system equilibration (which ranged from 0.6–1.2 s in different experiments), the rates of O2 uptake and H* ejection were recorded. Points taken from the traces at regular intervals were converted into digital form and used to determine rate law adherence, rates at successive time intervals, and extrapolation of the rates to zero time, taken as approximating the point of level flow (11, 18).

**RESULTS**

H*/O Ratio for Sites 2 + 3 from Measurements on Succinate Oxidation. Fig. 1 shows the electrode traces recorded from a typical experiment in which the rates of O2 uptake and H* ejection by AS30-D mitoplasts were measured. The test system (10°C) contained rotenone to prevent electron flow from site 1, valinomycin to allow K+ to serve as charge-compensating permeant cation, and N-ethylmaleimide to prevent H+ reuptake by symport with P¡ (10). With all other components present in the anaerobic medium the reaction was initiated by rapid injection of a precisely known amount of O2. Following a “dead period” owing to mixing and electrode system equilibration (which ranged from 0.6–1.2 s in different experiments), the rates of O2 uptake and H* ejection were recorded. Points taken from the traces at regular intervals were converted into digital form and used to determine rate law adherence, rates at successive time intervals, and extrapolation of the rates to zero time, taken as approximating the point of level flow (11, 18).

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Fig. 3. Vectorial $H^+/O$ ratio for succinate oxidation by mitoplasts from Ehrlich ascites tumor cells. The reaction conditions were precisely as in Fig. 1, but with 3.0 mg of mitoplast protein. The amount of oxygen injected was 31.6 nmol indicated by the horizontal curve. Data were taken from the unsmoothed traces at 0.2 s, converted into digital form, and tested for fit to different rate laws. The rates of both $H^+$ ejection and $O_2$ uptake were closely fitted by a simple exponential relationship, yielding a linear semilog plot as in Fig. 2. Zero time rate of $H^+$ ejection was 47.63 nmol/s and of oxygen uptake was 6.16 nmol oxygen/s, to give an $H^+/O$ ratio extrapolated to zero time of 47.63/6.16 = 7.73.

The traces show that $H^+$ ejection begins immediately and proceeds to a peak occurring slightly before the complete consumption of the added $O_2$. Thereafter rapid net reuptake of $H^+$ occurred via leaks. The half-time for back-leakage of $H^+$ was approximately 29 s, compared to about 10 s for attainment of the peak of $H^+$ ejection.

Fig. 2A shows the linear adherence of $\ln O_2$ versus time, corresponding to a first-order dependence on $O_2$ concentration, presumably owing to the increasing resistance or load in the form of $\Delta pH$ across the membrane (11, 21). Fig. 2, B and C shows the time dependence of the rates of $H^+$ ejection ($dH^+$/dt) and $O_2$ uptake ($-dO_2$/dt) as well as the extrapolation of these rates to zero time, the instant at which the load on electron flow is zero and level flow is presumed to exist. The extrapolation to zero time of the rates of $H^+$ ejection was 30.35 nmol $H^+/s$, equivalent to 674.4 nmol $H^+/min/mg$. The corresponding value of $O_2$ uptake was 3.93 nmol $O_2/s$ or 87.4 nmol $O_2/min/mg$ at 10°C. The observed $H^+/O$ flow ratio at zero time is, therefore, 30.35/3.93 = 7.72, typical of a series of experiments in which the extrapolated $H^+/O$ ratios closely approached 8.0.

It will be noted that the observed $H^+/O$ ratio for the succinate oxidation reaction declines sharply with time owing to $H^+$ backflow which evidently begins at zero time and becomes higher in rate as $\Delta pH$ increases. From the plots in Fig. 2, the $H^+/O$ ratio at 2 s was 6.66 and at 4 s was 5.48.

Fig. 3 shows the electrode traces obtained from an almost identical measurement of the $H^+/O$ ratio for succinate oxidation obtained on mitoplasts from Ehrlich ascites cells. The $H^+/O$ flow ratio obtained from the rates extrapolated to zero time in this experiment was 7.73. The observed $H^+/O$ ratio also declined with time similar to the case of the AS30-D mitoplasts.

Vectorial $H^+$ Translocation Coupled to Oxidation of Cyt c (+2). Fig. 4 shows the course of vectorial $H^+$ ejection and $O_2$
uptake by AS30-D mitoplasts in a system similar to that in Fig. 1 but in which succinate was replaced with 88.2 µM Cyt c (+2) mitoplasts were added at 3.07 mg protein. The reaction was initiated by injection of O2 into the anaerobic system and the changes in O2 and H+ recorded. Uptake of O2 was again nonlinear with time, as was H+ ejection. After the peak of H+ ejection was reached, net uptake of H+ began. The changes in O2 and H+ concentration with time at successive 0.1-s intervals were obtained from the traces in digital form and tested for best fit to different rate laws. The rate of O2 uptake gave a close fit to a simple exponential up to 2.5 s, as shown graphically in Fig. 5B. The zero time represents the moment the recorder shows the initial increase in O2 concentration. The reaction was monotonic until about 65% of the O2 had been consumed; thereafter the O2 uptake rate assumed a more complex relationship with O2 and ferrocytochrome c concentration, owing not only to the increasing ΔpH gradient across the membrane but also the competition of the Cyt c(+3) formed with Cyt c(+2) for binding to cytochrome oxidase. The net rate of H+ ejection was very high at the beginning and also declined exponentially but with a higher rate constant, as expected because the rate of H+ backflow across the membrane increases as the reaction proceeds, as ΔpH, the driving force for H+ backflow, increases with time. Extrapolation of the exponential H+ and O2 rates to zero time was carried out by computer as is shown graphically in Fig. 5. The plots yielded a zero time rate of H+ ejection of 54.96 nmol H+*/s and of O2 uptake of 15.12 nmol O2/s, corresponding to an H+/O2 flow ratio of 3.63 at zero time. Fig. 5 shows that the observed H+/O ratio declines exponentially and rapidly with time; at 1.0 s after the beginning of the reaction it had declined to 2.23 and at 2.0 s it was only 1.32, owing to the increasing rate of backflow of H+ as the reaction proceeds. The rate of decline of the H+/O ratio with time is considerably faster than in the case of the succinate H+/O ratio (Fig. 2).

Fig. 6 shows the traces recorded from a typical measurement of the H+/O ratio during oxidation of Cyt c(+2) by Ehrlich mitoplasts. The data were processed exactly as in Fig. 5 (plots...
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Fig. 8. Vectorial H*/O ratio for the cytochrome oxidase reaction in Ehrlich ascites tumor mitoplasts initiated by addition of ferrocytochrome c. Data taken from the unsmoothed traces in Fig. 7 at 0.1-s intervals were converted into digital form and line fitted and extrapolation to zero time carried out by computer. The rate of O2 uptake was first order up to 70% of its total extent.

Fig. 9. Measurement of the vectorial H* ejection coupled to the cytochrome oxidase reaction initiated by addition of ferrocytochrome c in AS30-D ascites tumor mitoplasts. The reaction conditions were precisely as in Fig. 7 but with 3.26 mg of mitoplast protein. The amount of oxygen added was 31.6 nmol. cyt c2+, Cyt c(+2), not shown) and yielded extrapolated zero time rates of 37.56 nmol H*/s and 10.71 nmol O2/s, which yields an H*/O ratio of 3.51. The rate of decline of the observed H*/O ratio for cytochrome oxidase in Ehrlich mitoplasts also exceeded that for succinate oxidation, as in the case of the AS30-D measurements.

Another experimental design to measure the cytochrome oxidase activity for the determination of the vectorial H*/O ratio is to initiate respiration by adding ferrocytochrome c to a system already containing O2. This procedure minimizes the dead period, i.e., the time required for mixing, electrode response, and recorder response. Fig. 7 shows the complete time course of an experiment in which the cytochrome oxidase activity from Ehrlich ascites tumor mitoplasts was initiated by the addition of ferrocytochrome c (12). Each step is described in the legend, including details of electrode calibrations.

The reaction was initiated by injection of ferrocytochrome c. O2 uptake began almost immediately and was accompanied by net H* ejection, which reached a maximum before completion of O2 consumption, showing that H* back-leakage proceeded at an increasing rate that soon equaled and then exceeded the declining rate of H* ejection as oxidation of the ferrocytochrome c neared completion.

The changes in O2 and H* concentration with time at successive 0.1-s intervals were obtained from the traces in digital form and used in a Guggenheim plot as is shown in Fig. 8. The rate of O2 uptake gave a close fit to a simple exponential up to 1.3 s, as shown graphically in Figure 8B. The reaction was monotonic until about 75% of the O2 had been consumed. The net rate of H* ejection was very high at the beginning and declined exponentially with time as in O2-pulse experiments (Fig. 6). Extrapolation of the exponential H* and oxygen traces to the zero time (set by the amount of ferrocytochrome c injected) yielded a rate of H* ejection of 80.75 nmol H*/s and of O2 uptake of 24.47 nmol oxygen/s, corresponding to an H*/O ratio of 3.30 at zero time.

Fig. 9 shows the same type of reductant pulse experiment performed with AS30-D mitoplasts. The traces of H* ejection and O2 uptake followed the same pattern as in Ehrlich ascites mitoplasts. Fig. 10 shows extrapolations of the exponential H* and O2 rates to zero time. The plots yielded an initial rate of H* ejection of 85.20 nmol H*/s and of O2 uptake of 23.84 nmol O2/s.
nmol O/s, corresponding to an H+/O flow ratio of 3.57 at zero time.

Table 1 shows the vectorial H+/O translocation ratios characteristic of energy-conserving sites 2 + 3 and site 3 of mitoplasts from AS30-D and Ehrlich ascites tumor cells. The H+/O ratios in both tumor lines exceeded 7.2 for succinate oxidation and 3.3 for cytochrome c oxidation whether the reaction was initiated by a pulse of O₂ or a pulse of ferrocytochrome c, suggesting very strongly that the values of the H⁺ per site ratio are close to 4.0, in close agreement with data of normal mitoplasts (12, 21). The rates of H⁺ back-decay in tumor mitoplasts are, however, relatively higher. Moreover, the rates of O₂ uptake and H⁺ ejection estimated with the cytochrome c-pulse method were apparently higher than those obtained with the O₂ pulse due, in all probability, to a fast rate of formation of oxidized cytochrome c (an inhibitor of the cytochrome oxidase reaction) during the first 500 ms of the oxygen pulse experiment (28).

**DISCUSSION**

The measurements reported here, carried out with "second-generation" methods involving a greatly improved O₂ electrode matched in response to the pH electrode together with extrapolation of the rate measurements to zero time, show that for mitoplasts from AS30-D and Ehrlich ascites cells the observed H⁺/O ratio of succinate oxidation approaches 8.0 and that of the cytochrome oxidase reaction approaches 4.0. The data on succinate oxidation confirm earlier measurements, made with less accurate methods, reported from this laboratory (4). Since all reported values for the H⁺/2e⁻ ratio for site 2 alone is 4, in both normal and tumor preparations (cf. Ref. 5), the H⁺/O ratio of close to 8 for sites 2 + 3 provides an important check made with the natural substrate succinate on the validity of using Cyt c (+2) as electron donor for direct tests on site 3, as described in this paper. These data also show that the observed H⁺/O ratios for sites 2 + 3 and site 3 alone in these 2 tumor cell lines are similar to those of normal rat liver (11, 21), as was concluded earlier (29).

Again, it was observed that the rate of decline of the observed H⁺/O ratio for cytochrome oxidase was greater than the decline of the observed H⁺/O ratio for succinate oxidation, as is the case in normal mitoplasts. This observation suggests that the rate of H⁺ back-decay may depend in part on the specific segment of the electron transport chain through which electron flow occurs.

While the identity of the corresponding zero time H⁺/O ratios between normal liver and heart versus the 2 tumor cell lines examined may suggest that there is no particular difference in the efficiency of energy transduction of malignant cells, this is perhaps a premature conclusion for 2 reasons: (a) it has been observed here and in an earlier study (29) that the rate of H⁺ back-decay is considerably higher in tumor mitochondria or mitoplasts than in normal tissue preparation (12, 21), which could be expected to cause a significant deficit in the net efficiency of electron transport; (b) electron transport in intact cells is almost certainly not proceeding with H⁺/O ratios close to the mechanistic, since the respiration of many intact cells is close to being in state 4, in which leak rates tend to be near maximal. Earlier observations made in this laboratory (30) and elsewhere (31) strongly suggest that oxidative phosphorylation is less efficient in tumor cells than in normal tissues.

Experiments are under way in an effort to determine on a more quantitative basis the intrinsic rates of H⁺ backflow through the inner membrane of mitochondria and the actual efficiency of energy transduction in normal and tumor cells.

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