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MITOCHONDRIAL FUNCTION UNDER HYPOXIC CONDITIONS THE STEADY STATES OF CYTOCHROME $a+a_3$ AND THEIR RELATION TO MITOCHONDRIAL ENERGY STATES

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SUMMARY

The oxidation–reduction states of mitochondrial cytochromes were studied under low O_2 concentrations

1 Relative oxidation states of cytochromes caused by increasing concentrations of O_2 apparently followed the sequence of their half reduction potentials only under the uncoupled conditions

2 In the presence of antimycin A, the O_2-induced reduction of cytochrome b_T was seen in the difference spectrum

3 The O_2 dependency of the relative reduction state of cytochrome $a+a_3$ with respect to that of cytochrome c altered significantly depending upon the presence or absence of ATP The most significant change in the O_2 dependency was that due to cytochrome a_3

4 When compared at a given low O_2 concentration below 0 5 μM, the reduction states of cytochrome $a+a_3$, as well as that of cytochrome c, were higher in the presence of ADP or uncoupler than in the presence of ATP

5 Whereas the O_2 concentration required for 50 % oxidation of cytochrome c ($P50_c$) depended upon the respiratory rate, the O_2 concentration required for 50 % oxidation of cytochrome a_3 ($P50_{a3}$) required information on the energy state of mitochondria Under conditions where the redox states of cytochrome c and $a+a_3$ are measured continuously and as a function of the O_2 concentration it may be possible to evaluate the energetic state of the mitochondria

INTRODUCTION

Applications of spectrophotometric and fluorometric techniques to perfused organs [1–4] and even to the organs of anaesthetized animals [5, 6] are appropriate

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procedures for the pursuit of complex biochemical reactions in intact organs under physiological conditions One successful example is the spectrophotometric analysis of the steady state of catalase–H_2O_2 compound in perfused rat liver [7, 8], by which the profile of cellular H_2O_2 metabolism was quantitatively studied [8–10] Quantitation of pyridine nucleotide fluorescence as an indicator of the cytoplasmic redox state in perfused heart and liver was accomplished respectively by Williamson and Jamieson [2] and by Bucher and co-workers [11], this method reinforced the usefulness of this technique in the study of intact organ systems

Previously [12], we attempted to quantitate the use of the redox states of mitochondrial cytochrome *c* and pyridine nucleotides as indicators of tissue hypoxia Regardless of mitochondrial metabolic conditions, the O_2 concentration required for 50 % oxidation of cytochrome *c* ($P50_c$) was found to decrease approximately linearly with the decrease of electron flow rate through the respiratory chain [12] Whereas cytochrome *c* is only an electron carrier, cytochromes of *b* type and $a+a_3$ in the respiratory chain appear to be involved in the energy coupling mechanism [13, 14] and may be used as indicators of cellular energy state in tissues

This paper investigates specifically the redox response of cytochrome $a+a_3$ to various O_2 concentrations and provides data on their characteristic profile as a function of the energy-coupling activity of the mitochondria

MATERIALS AND METHODS

Spectrophotometry

The difference spectra of varying degrees of hypoxic minus anaerobic or hypoxic mitochondrial suspensions were measured in a single cuvette, using a dual-wavelength scanning spectrophotometer which was connected to a computer as reported previously [15, 16] The simultaneous measurements of the redox states of cytochrome $a+a_3$ (445–450 nm, or 605–620 nm), of cytochrome *c* (550–540 nm) and of the O_2 concentration were carried out in a single cuvette, using a combination of a time-sharing dual wavelength spectrophotometer [17] and a fluorometer [12, 18]

O_2 titration

As an indicator of the O_2 concentration, *Photobacterum phosphoreum*, which was obtained through the courtesy of Dr F H Johnson, Princeton University, was used [18] The procedure of a steady-state titration with O_2 of the oxidation–reduction states of cytochrome $a+a_3$ and cytochrome *c* was reported in detail previously [12, 18] The reaction mixture consisted of 0 5 M mannitol, 0 1 M KCl, 0 01 M potassium phosphate (pH 7 2) and 0 01 M glucose ('high chloride' medium [12]) When desired, respiratory substrates, magnesium chloride (2 mM), ATP-generating system, ADP-generating system or other chemicals were supplemented In some experiments, 'standard' medium (0 225 M mannitol, 0 075 M sucrose, 0 02 M morpholinopropane sulphonate (pH 7 2), 2 mM KCl, and 0 2 mM EDTA) was also used The results obtained in both media were almost identical

Other procedures

Pigeon heart mitochondria were prepared according to the method of Chance and Hagihara [19] Pyruvate kinase (14 I U /ml) was used with 3 mM phosphoenol

pyruvate and 0 5 mM ATP as an ATP-generating system For an ADP-generating system, hexokinase (10 I U /ml) was used with 10 mM glucose, 5 or 10 mM potassium phosphate, and 0 5 mM ADP Protein was determined by the Biuret method using bovine serum albumin as a standard

Materials

Hexokinase and pyruvate kinase were purchased from Sigma Chemical Co , St Louis, Mo , U S A Ultra-pure argon (99 999 %) was obtained from Matheson Gas Products, Gloucester, Mass , U S A

RESULTS

Difference spectra of mitochondrial cytochromes produced by hypoxic conditions

Prior to a detailed examination of the O_2 dependency of pigeon heart mitochondria by dual wavelength spectrophotometry, it is appropriate to describe the absorption spectra of mitochondrial cytochromes produced by various degrees of hypoxia The anaerobic spectrum of pigeon heart mitochondria in the presence of uncoupler (pentachlorophenol) and antimycin A was memorized by the computer associated with the spectrophotometer and was fed back as time-shared dynode voltage corrections to give the flat base line in Fig 1 The anaerobic suspension was then stirred with a magnetic stirrer under a continual argon gas stream This treatment results in a continual introduction of very low concentration of O_2 into the suspension and allows a steady free O_2 concentration below 0 005 μM A reduction of *b* type cytochrome was observed, which was identified to be cytochrome b_T [13, 20] by its double α-peak around 567 and 558 nm, shoulder β-band at 538 nm and the Soret band at 432 nm [16] Such a reduction of cytochrome *b* by introduction of O_2 was recorded by kinetic measurement under similar conditions [21] Cytochrome oxidase is slightly oxidized as shown by the α- and Soret bands As the oxygen concentration is increased in a stepwise fashion by increasing the proportion of O_2 in the gas stream, cytochrome oxidase and cytochrome *c* become progressively oxidized

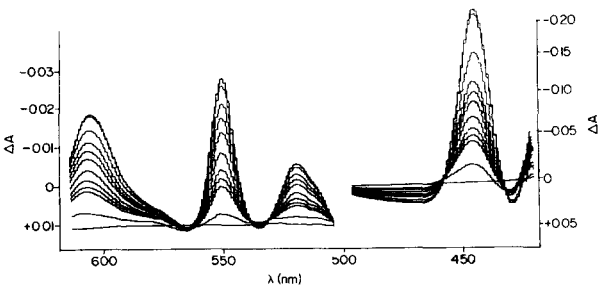

Fig 1 Difference spectra of various degrees of hypoxic minus anaerobic mitochondria in the presence of antimycin A and uncoupler The reaction mixture consisted of 0 225 M mannitol, 0 075 M sucrose 0 02 M morpholinopropane sulfonate (pH 7 2), 2 mM KCl 0 2 mM EDTA 6 mM succinate 6 mM glutamate, 0 8 mg/ml pigeon heart mitochondria, 0 5 μg/ml antimycin A, and 10 μM pentachlorophenol A spectrum of anaerobic mitochondrial suspension was recorded as a flat base line and the difference spectra produced by introduction of different concentrations of O_2 was subsequently recorded as described previously [12] Reference wavelength used was 540 nm

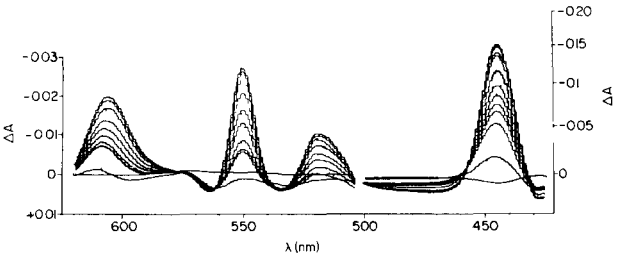

Fig 2 Difference spectra of various degrees of hypoxic minus anaerobic mitochondria in the presence of antimycin A and ATP The reaction mixture was as described in Fig 1, except that the mitochondrial concentration was 0 5 mg/ml and, instead of uncoupler, an ATP-generating system (0 5 mM) was added Reference wavelength used was 575 nm

When the uncoupler is omitted and an ATP-generating system is included together with antimycin A (Fig 2), the spectral changes in response to increasing O_2 concentrations differ from those observed in the uncoupled conditions In addition to the reduction of cytochrome b_T, significant oxidation of cytochrome oxidase is seen at an O_2 concentration below 0 005 μM The difference spectrum observed in the α-band of cytochrome oxidase at this stage shows a change in the position and shape of the trough The relative oxidation of cytochrome oxidase and cytochrome c in a following titration with oxygen is also different under the ATP-supplemented and the uncoupled conditions (see below)

In the absence of antimycin A, the redox state of b-type cytochromes are more complicated and, thus, individual difference spectra corresponding to each step of the titration with oxygen were recorded as shown in Fig 3 The difference spectrum observed with the O_2 concentration below 0 005 μM (row A) in the presence of uncoupler is that of an oxidized-minus-reduced cytochrome b_T [16], as indicated by the trough of the double α- and the Soret bands No detectable oxidation in the other cytochromes is noticed in this condition This spectrum, in turn, was recorded as a

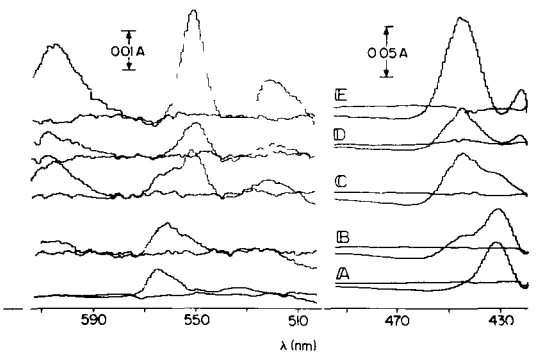

Fig 3 Difference spectra of mitochondrial cytochromes under various degrees of hypoxic conditions The conditions were as described in Fig 1, except that antimycin A was omitted and the mitochondrial concentration was 1 1 mg/ml O_2 concentrations in the medium were subsequently increased in a sequence from A to E (100 % O_2) At each step the difference spectrum was re-memorized into a computer so that the contribution of this spectrum was nullified in the spectrum of the next step, as indicated by flat base lines in each row

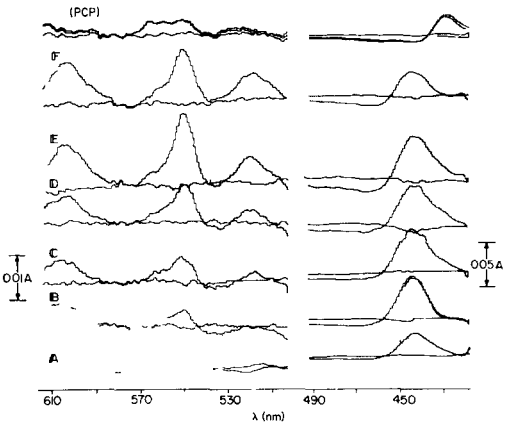

Fig 4 Difference spectra of mitochondrial cytochromes under various degrees of hypoxic conditions The conditions were as described for Fig 3, except that substrate was glutamate (6 mM) only, and the mitochondrial concentration was 1 0 mg/ml The spectrum presented in row G was that produced by adding pentachlorophenol under 100 % O_2

flat base-line in row B, and the O_2 concentration was further increased to approximately 0 02 μM The cytochromes which are oxidized at this stage are cytochrome b_K (α-trough at 562 nm and the Soret trough at 428 nm [16]) and cytochrome $a+a_3$ The oxidation of cytochrome *c* becomes obvious at the third step (row C) It appears that the relative degrees of oxidation of mitochondrial cytochromes (except for cytochrome a_3) in response to the change in the O_2 concentration, apparently follow the relative values of their midpoint potentials (cytochrome b_T, -30 mV, cytochrome b_K, $+30$ mV, cytochrome *c*, $+235$ mV, cytochrome *a*, 210 mV, and cytochrome a_3, 385 mV) [14, 22] under the uncoupled conditions

In comparison, the difference spectra obtained in the absence of uncoupler and in the presence of glutamate only, are shown in Fig 4 A portion of *b*-type cytochromes is oxidized together with cytochrome $a+a_3$ at the lowest O_2 concentration (row A), but another portion remains in the reduced state initially and is gradually oxidized as the O_2 concentration increases (rows B–F) In this experiment, only an 80 % reduction of *b*-type cytochromes, when measured at 560 nm, was achieved under anaerobic conditions with glutamate alone

Relative oxidation–reduction states of cytochrome $a+a_3$ and cytochrome c under hypoxic conditions

With the aid of the difference spectra presented above, it is now possible to identify the components being measured by dual wavelength spectrophotometry and to plot the redox states of the cytochromes as a function of the oxygen concentration In Fig 5, the oxidation–reduction states of cytochrome $a+a_3$ measured at 445–450 nm are compared with those of cytochrome *c* at various O_2 concentrations in the presence of antimycin A With the ATP-generating system, approximately 50 % of cytochrome $a+a_3$ is more sensitive to O_2 than is cytochrome *c*, the remainder behaving in a way similar to cytochrome *c* In contrast to this, when an uncoupler is present, 50 % of cytochrome $a+a_3$ becomes less sensitive and, thus, higher O_2

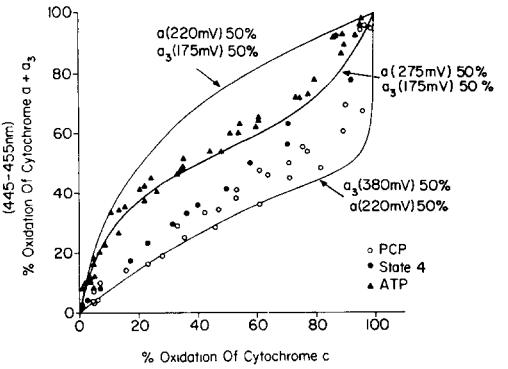

Fig 5 Relative oxidation–reduction state of cytochrome $a+a_3$ with respect to that of cytochrome c in the presence of antimycin A The conditions were similar to those described in Figs 1 and 2 Wavelength pairs used for cytochrome $a+a_3$ and cytochrome c were 445–450 nm and 550–540 nm, respectively ▲, in the presence of ATP and its generating system, ○, in the presence of pentachlorophenol, ●, with succinate plus glutamate alone Solid lines represent relative redox states of cytochrome $a+a_3$, calculated based on the half-reduction potentials of cytochromes $a+a_3$ and c For details see Discussion

concentrations are required to produce oxidation Without adding ATP or uncoupler, an intermediate profile is obtained in this plot The O_2 concentration required for 50 % oxidation of cytochrome c in the presence of antimycin A was approximately 0 04 μM [12], and the respiratory rate at this oxygen concentration was below 4 nmoles O_2 per min per mg mitochondrial protein One of the components in cytochrome oxidase exhibits a remarkable change in its O_2 dependency between the ATP-supplemented and uncoupled conditions This is probably cytochrome a_3, as seen also in the spectral change of the α-region shown in Fig 2 This spectral shift was interpreted to be attributable to that associated with the change in the half-reduction potential of cytochrome a_3 by Lindsay and Wilson [23]

In the absence of antimycin A, a different relationship between the relative oxidation–reduction states of cytochrome $a+a_3$ and of cytochrome c is obtained (Fig 6) As previously reported [24, 25], cytochrome a_3 is not significantly reduced by succinate-plus-glutamate under anaerobic conditions if the system contains ATP This observation was made by comparison of the redox state of cytochrome $a+a_3$ and H^+ release due to ATP hydrolysis associated with the aerobic–anaerobic transition [25] Here we observed that only approximately 15 % of cytochrome $a+a_3$ remained in the oxidized state under anaerobic conditions Obviously, an ATP/ADP ratio maintained by an ATP-generating system in the present study is lower than the value obtained by an extrapolation in the previous study [25] Nevertheless, the ATP-induced change is observable in the redox profile of cytochrome $a+a_3$ in response to the O_2 concentration, the difference between those observed with and without antimycin A is that, in the absence of antimycin A, ATP causes a more pronounced deviation of the relative reduction state of cytochrome $a+a_3$ from that of cytochrome c than it does in the presence of antimycin A With uncoupler, the experimental points distribute in a similar way to that seen in the presence of antimycin A Two components of cytochrome oxidase are distinguishable, the reduction state

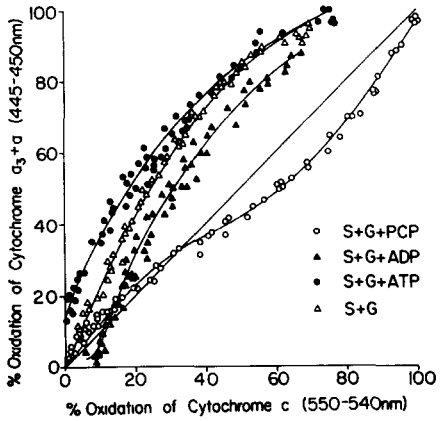

Fig 6 Relative oxidation–reduction states of cytochrome $a+a_3$ with respect to that of cytochrome c in the absence of antimycin A The conditions were similar to those described for Figs 3 and 4 △, with succinate-plus-glutamate, ○, with pentachlorophenol, ▲, with ADP (0 5 mM) and its generating system, ● with ATP (0 5 mM) and its generating system

of one component, probably of cytochrome a_3, is less sensitive to O_2 than those of cytochromes a and c Of interest is the observation that the redox behavior of cytochrome $a+a_3$ with an ADP-generating system differs from that with uncoupler, in a range of low O_2 concentrations, the curve shifts toward the right side in this plot, but, as the O_2 concentration increases, the curve tends to merge with the curve observed in the presence of ATP This difference suggests a direct influence of the state of the phosphorylation system on the redox state of cytochrome $a+a_3$ An intermediate curve is obtained with succinate plus glutamate A progressive transition of the state due to the increasing endogenous ATP/ADP ratio may be expected under this condition

Similar experiments were performed in 'high chloride' medium and the results are presented as a function of O_2 concentration in Fig 7 With ATP (A), the oxidase curve exhibits a clear biphasicity which is similar to that observed in respiring yeast cells [15] The component exhibiting a higher O_2 affinity is attributable to cytochrome a_3 Thus, cytochromes a and c may show apparently similar oxygen affinities (P50 = 0 1 μM) When ADP is present (B), the $P50_c$ is 0 27 μM The cytochrome oxidase curve is monotonic and the $P50_{a+a3}$ is 0 15 μM Under State-4 conditions, the curve is a mixture of the others as the titration progresses due to ATP formation and, thus, a slope of the curve is rather steep compared to the others Although the contribution of cytochrome a_3 absorbance was slightly smaller, similar results were also obtained at 605–620 nm

Effect of substrates on the redox behavior of cytochrome $a+a_3$

As shown in Fig 8, the titration curve obtained in the absence of substrate is similar to those observed under uncoupled conditions in Figs 5 and 6 As the respiratory rates are increased with either glutamate, β-hydroxybutyrate, or succinate, the reduction state of cytochrome $a+a_3$ becomes more sensitive to O_2 as compared with that of cytochrome c These results are given as a function of the O_2 concentration

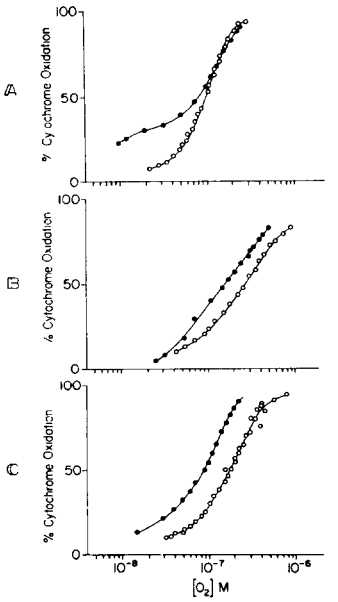

Fig 7 The relationship between the redox states of cytochrome $a+a_3$ and of cytochrome c and oxygen concentration The reaction medium was high chloride' medium as described in Materials and Methods O_2 concentrations were determined by the bioluminescence of *Photobacterium phosphoreum* A, with ATP-generating system, B, with ADP-generating system, C succinate plus glutamate alone, ○, cytochrome c, ●, cytochrome $a+a_3$

in Fig 9 In confirmation of previous results [12], the $P50_c$ is 0 27 μM with succinate-plus-glutamate, 0 07 μM with β-hydroxybutyrate, 0 06 μM with glutamate, and 0 035 μM with endogenous substrates, respectively These values were apparently proportional to the respiratory rates in corresponding conditions [12] Therefore, it is seen that, the faster the respiratory rate, the greater the difference between the values of

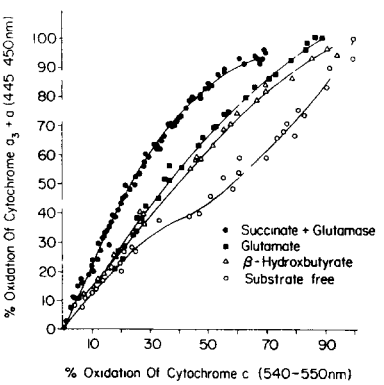

Fig 8 The effects of substrates on the relative redox states of cytochromes $a+a_3$ and c The conditions were as described in Fig 4 ■, with glutamate (6 mM), △, with β-hydroxybutyrate (2 mM), ○, without exogenous substrates, ●, succinate (6 mM) plus glutamate (6 mM)

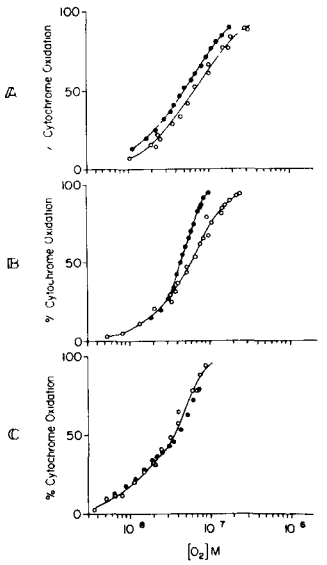

Fig 9 The relationship between the redox states of cytochrome $a+a_3$ and c and O_2 concentration in the presence of different substrates The conditions were as described in Fig 7 A, with β-hydroxybutyrate (2 mM), B, glutamate (6 mM), C, with endogenous substrates The result with succinate plus glutamate was given in Fig 7 ○, cytochrome c, ●, cytochrome $a+a_3$ ($A_{445-450\ nm}$)

$P50_c$ and $P50_{a+a3}$ The magnitude of these differences may be proportional to the free energy available from this redox couple to the phosphorylation system, and may control the respiratory rate Direct determination of the value of free energy available from the redox couple of mitochrondrial NADH and cytochrome c was attempted by Wilson et al [26] However, such determination is not feasible in the present system

DISCUSSION

The experimental data illustrates alterations in the response to oxygen of the redox states of cytochromes which in turn depend upon the bioenergetic state of the mitochondria The response of the redox state of cytochrome c as a function of O_2 concentration (Figs 7 and 9) is roughly hyperbolic (sigmoidal on a linear-log plot as employed here) The $P50_c$ is proportional to the electron flow rate through the respiratory chain [12]

Cytochrome $a+a_3$ and probably cytochrome b's exhibit more complex types of behavior which are the main topic of this contribution When these profiles are referred to that of cytochrome c, a deviation of proportionality between cytochrome c and cytochrome $a+a_3$ is exhibited at low O_2 concentrations These deviations are to the right in the presence of uncoupler and to the left in the presence of ATP (Figs 5 and 6) A variety of states between these extremes depend upon the degree to which the mitochondrial suspension, initially near State 3 due to the presence of phosphate and endogenous ADP, may shift to near State 4 during the titration, with increasing

concentrations of O_2 which increase the ATP/ADP ratios The interpretation of the results depends upon an identification of the absorbance change at 445–450 nm that is due to cytochrome a_3 or to cytochrome a Cytochrome a_3 is defined and identified by its ability to react with O_2 prior to cytochrome a [27] but such kinetic experiments are not feasible under the steady state conditions employed in the O_2 titration Wilson and co-workers [23] identified cytochrome a_3 as the component which exhibits an ATP-dependent midpoint potential change based on the effect of externally added ligands Consequently, cytochrome a_3 would be expected to exhibit a greater alteration of the profile of cytochrome oxidase absorbance against cytochrome c absorbance This identification can be confirmed as follows The respiratory rate (dO_2/dt) can be expressed by an equation

$$\frac{dO_2}{dt} = k_1 \, [a_3{}^{2+}] \, [O_2] \tag{1}$$

where k_1 is a second-order rate constant for the cytochrome $a_3{}^{2+}$–O_2 reaction. Schindler [28] measured the respiratory rates of pigeon heart mitochondria in the range of O_2 concentrations between 0 1 and 0 005 μM, using the bacterial luminescence method [18, 29] The values of $k_1[a_3{}^{2+}]$ with and without ATP or ADP were calculated from his data [28] and given in Fig 10A as a function of O_2 concentration. For comparison the absorbance changes at 445–450 nm, including the oxidation–reduction of both cytochromes a and a_3, were also replotted in Fig 10B A clear difference in the terms $k_1 \, [a_3{}^{2+}]$ which were calculated from the values of dO_2/dt and $[O_2]$ is seen between the presence of ATP and ADP Such a dependency of the absorbance change on ATP and ADP is seen after approx 50 % reduction of cytochrome $a+a_3$ Therefore, it is obvious from this comparison that the component changing its O_2 dependency by ATP is cytochrome a_3, or at least this is the molecular species directly participating in the reaction with O_2

According to Wilson et al [30], the half-reduction potentials (E_m) of cytochrome $a+a_3$ are 220 and 175 mV respectively in the presence of ATP, and 220 and 380 mV in the presence of uncoupler In a later work [23], they reported the ATP-dependent change of E_m values of cytochrome a from 220 to 275 mV The E_m value of

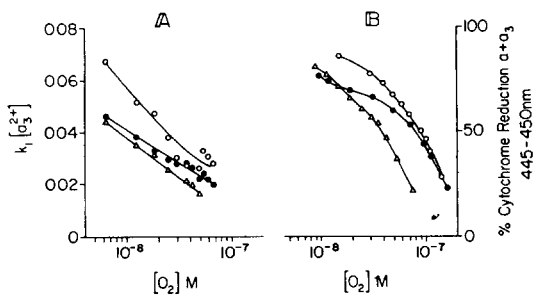

Fig 10 The redox states of cytochrome a_3 under low O_2 concentrations The values of $k_1[a_3{}^{2+}]$ under various O_2 concentrations in A were calculated from Dr Schindler's data [28], using an equation, $dO_2/dt = k_1 \, [a_3{}^{2+}] \, [O_2]$ For a comparison, the data presented in Fig 7 was re-plotted in B as absorbance change at 445–450 nm vs O_2 concentrations ○, with ADP, ●, with ATP, △, with endogenous substrate only

cytochrome c is 235 mV and is not dependent upon the energy state of the mitochondria [31] The thermodynamic properties of the redox couple can be described as an equation

$$\Delta E_{h\,(A-B)} = \Delta E_{m\,(A-B)} + (RT/nF)\,(1n\,\frac{[A_{ox}][B_{red}]}{[A_{red}][B_{ox}]}) \tag{2}$$

Assuming that the respiratory system functions in near equilibrium [14, 32], even when O_2 is at limiting conditions, ΔE_h between the redox couple of cytochrome a and c is zero When the electron transfer reaction is coupled with the phosphorylation reaction, the definition of an approach to equilibrium is that the summation of the free energy changes of the coupled reactions approaches zero (see refs 14 and 22) Therefore, when considering the redox couple of cytochrome a_3 and c, ΔE_h is zero in the uncoupled condition, but not zero under other conditions, and is equivalent to the ΔE available for phosphorylation reaction

$$\Delta E = -\frac{\Delta G'_{oATP}}{nF} - (RT/nF)\,(1n\,\frac{[ADP][P_i]}{[ATP]}) \tag{3}$$

Since the $[ATP]/[ADP][P_i]$ ratio was known in the initial condition, but was not determined throughout the course of the titration experiments, precise applications of these equations was not possible However, the relative redox states of cytochrome c and cytochrome $a+a_3$ were calculated based on the Eqn 2 and were given in Fig 5 (solid lines) Assuming that the proportion of cytochrome a_3 absorbing at 445–450 nm is 50 % [23], and that the E_m values for cytochromes c, a and a_3 are 235, 220 and 380 mV respectively [30] under uncoupled conditions, the curve obtained fits the experimental data For the system including ATP, the E_m value of cytochrome a was assumed to be 220 mV [30], and a potential change of cytochrome a_3 either in terms of E_m or E_h was assumed to be −205 mV This difference corresponds to the ΔE_m value observed in the 'potential clamped' system with ATP (change from 380 to 175 mV) [23, 30] The curve thus obtained fits the experimental data only in the low O_2 concentration range However, the calculated value fits the data over a wider range if the change in the E_m value of cytochrome a in the presence of ATP is also assumed to be from 220 to 275 mV [23] These results, therefore, indicate that the redox changes induced by ATP under low O_2 concentrations are explainable in terms of ΔE_h by the changes in the midpoint potentials of both cytochromes a and a_3 The fit was good only for the conditions where the respiration was heavily inhibited by the presence of antimycin A and by low O_2 concentrations When the respiratory rate was faster, the deviation of the oxidase curve from that of cytochrome c became greater (Figs 6, 7, 8 and 9) However, the assumption of equilibrium may be even more severely violated at these electron flow rates

This series of studies [12, 25] has been carried out with the intention of applying the results to the analysis of intact organ systems where direct, spectrophotometric measurements of intracellular O_2 concentration and of bioenergetic states are of particular interest By using cytochromes as an indication of tissue bioenergetic states, the deviation of the O_2 dependency of cytochrome a_3 from that of cytochrome c may be utilized Such a deviation (Fig 7A) was indeed seen in highly respiring yeast cells [15], which may indicate a relatively high ATP/ADP ratio in the cell In contrast to

this observation, no clear deviation was detected in the perfused heart (M Tamura, unpublished) In the latter system, the energy demand for the contraction–relaxation of heart beat may result in the cyclic alteration of the ATP/ADP ratio This cyclic alteration in ATP/ADP ratio appeared to cause a cyclic fluctuation of the redox state of cytochrome a_3 which, in turn, further relates to the changes in the redox states of other cytochromes, and also to the changes in the respiratory rate and intracellular O_2 concentration Since tissue O_2 tension can be monitored by the oxygenation state of tissue myoglobin, cyclic alteration occurring in the beating heart may be analyzed in terms of its bioenergetic states, based on the mode of change in the redox states of cytochrome $a+a_3$ and of cytochrome c This project is now in progress [33]

Slater and his colleagues have provided a number of experiments suggesting that the cytochrome oxidase–O_2 reaction can be reversed by ATP, contrary to the previous results of Schindler as quoted by Chance [34] The available preliminary publications put forward two lines of evidence One, based on the oxy-graph and the second, based upon an O_2-assay method different from ours In the case of the oxy-graph trace, the "breakpoint" of the oxy-graph trace (Fig 1, p 644 in ref 35) shows a change dependent upon ATP This change is attributed to a change in the dissociation constant of oxygen from cytochrome oxidase This might be due to a change of electron flow rate as well However, the oxy-graph has been seriously criticized as being unsuitable for determining the K_m for O_2 of cytochrome oxidase [36, 37] Starlinger and Lubbers [36] conclude that the critical P_{O2} as revealed by the oxy-graph is dependent upon the properties of the oxy-graph itself and not upon the mitochondria Their further work indicated that the O_2 pressures appropriate to mitochondrial function (0 05 Torr O_2 pressure) are below the range which can be measured with the polarographic method [37] Thus, we maintain that the O_2 affinity in the presence and absence of ATP is best determined with the luminescent system

The second approach, namely, to determine by an O_2 assay system the production of O_2 from mitochondria gives negative results when using intact cells as has Schindler and as have we in this paper On the other hand, when subcellular particles are used as from *Acetobacter mesoxydans* positive results are obtained [38] The differences in experimental conditions, namely, whole cells on the one hand and subcellular particles on the other hand raises the question of whether or not the subcellular particles can respond to direct electron transfer between the mitochondria and the particles, a reaction which is not possible between the mitochondria and the whole cells It is therefore appropriate to reserve judgement as to whether there is a real discrepancy between the two approaches until data are available with Bienfait's method using intact cells Under these conditions, the CO sensitivity should be demonstrable

In summary, it appears that the assay methods which respond only to dissolved O_2 and are sensitive to the region of 10^{-9} M are essential for the quantitative study of the reactions of cytochrome oxidase with O_2

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