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MITOCHONDRIAL OXYGEN AFFINITY AS A FUNCTION OF REDOX AND PHOSPHATE POTENTIALS

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SUMMARY

1. The conditions under which mitochondria might catalyse a net reversal of oxidative phosphorylation are analysed.

2. Rat-liver mitochondria, incubated under such conditions, show a strongly diminished affinity for oxygen.

3. The velocity of respiration under these conditions is a hyperbolic function of the oxygen concentration.

4. The K_m for oxygen is less than 0.1 μM at low phosphate potential, irrespective of substrate, and 1–3 μM under reversal conditions.

5. The observed kinetics can be accounted for in a simple mechanism for cytochrome oxidase action.

INTRODUCTION

Respiratory-chain phosphorylation, although its mechanism is not yet fully understood, is generally agreed to be an enzymic process. This means that the enzyme machinery, the mitochondrial respiratory chain, facilitates the interaction between the reaction components, but does not influence the equilibrium constant of the overall reaction.

A simple description of this reaction is:

$$SH_2 + \tfrac{1}{2}O_2 + n\,ADP + n\,P_i \rightleftharpoons S + n\,ATP + (n-1)H_2O \qquad (1)$$

in which the value of n depends on the nature of S.

The most important physiological role of the process is to produce a high ratio of [ATP]/([ADP] · [P_i]), or a high phosphate potential*, so that the cell has at its disposal energy at a sufficiently high intensity to drive its energy-requiring processes.

Abbreviation: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

* By 'phosphate potential' is meant the absolute value of ΔG of the ATPase reaction, i.e. it is equal to $\Delta G_0 + RT\ln$ [ATP]/([ADP] · [P_i]), where ΔG_0 is the standard change of free energy for the hydrolysis of ATP to ADP and P_i.

In the presence of the necessary reactants, isolated mitochondria can load this phosphate potential up to a value of 67 kJ [1]. Such a high phosphate potential is of course possible only when the energy coming from the oxidation process

$$SH_2 + \tfrac{1}{2}O_2 \rightleftharpoons S + H_2O \qquad \Delta G_0 = -A \text{ kJ} \tag{2}$$

is not used for the phosphorylation of too many ADP molecules in the phosphorylation reaction

$$ADP + P_i \rightleftharpoons ATP + H_2O \qquad \Delta G_0 = +B \text{ kJ}[2] \tag{3}$$

If, for example, the oxidation of succinate by oxygen were coupled to the phosphorylation of three molecules of ADP, instead of two as found experimentally, the phosphate potential could not reach this value, as a simple calculation will show. The $-\Delta G_0$ for the oxidation of succinate by oxygen is 160 kJ and it would be difficult for the $-\Delta G$ value in vivo to reach more than 170 kJ, which could sustain a phosphate potential of maximally 57 kJ. If a phosphate potential of 67 kJ were introduced into the system, the reaction given in Eqn 1 would be reversed until the phosphate potential fell to 57 kJ.

A consequence of the organisation of the respiratory chain is that, under normal experimental conditions, the reaction given in Eqn 1 is always seen to go to the right. Partial reactions of the enzymic machinery, the so-called Sites 1 and 2, have been shown to be reversible [3–5], but not the overall reaction given in Eqn 1. This has given rise to a belief that this reaction is irreversible. However, since the enzyme machinery cannot alter equilibrium constants, the reverse reaction must also be catalysed by mitochondria, only we do not know how rapid this reaction will be. More knowledge on this point might be useful for our insight into the mechanism of oxidative phosphorylation especially at Site 3.

The aim of this investigation is to test the validity of the above considerations, by determining the conditions most favourable for reversal of respiratory-chain phosphorylation, and to study the mitochondrial oxygen metabolism under those conditions.

MATERIALS AND METHODS

Rat-liver mitochondria were isolated as described by Myers and Slater [6]. Protein was determined according to Cleland and Slater [7]. Oxygen concentrations were measured with a Gilson Medical Electronics Oxygraph equipped with a Clark electrode. The all-glass reaction vessel had a volume of 1.6 ml and was thermostated at 25 °C. A glass stopper with a narrow capillary limited oxygen diffusion to a maximal value of about 0.2 μM/min. In some cases extra care was taken against diffusion by bathing the joints of the vessel in fresh dithionite solution and keeping the end of the glass stopper in a constant stream of oxygen-free nitrogen. Under these circumstances no diffusion of O_2 could be demonstrated in 30 min.

Redox levels of cytochromes were measured with an Aminco-Chance dual-wavelength spectrophotometer. The 4-ml quartz vessel had molten joints and was closed with a glass stopper with narrow capillary. This cuvette showed no diffusion of oxygen, the criterion being oxidation of NADH by *Acetobacter mesoxydans* particles. The wavelength pairs used were 445/455 nm for cytochromes $a + a_3$, and 550/540 nm for cytochromes $c + c_1$.

The standard reaction medium contained 24 mM Tris · HCl buffer, 25 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA and 0.5 mM potassium phosphate. The pH, unless otherwise mentioned, was 7.4. The temperature of the spectrophotometer experiments was about 22 °C.

A strain of *A. mesoxydans* was kindly given by Dr J. G. van Andel of the Laboratory for Microbiology of the University of Amsterdam. The bacteria were kept in Petri dishes on a medium containing 1.5 % agar, 1 % yeast extract, 3 % $CaCO_3$ and 0.2 M ethanol. 2-l liquid cultures were grown in continuously shaken 5-l Erlenmeyer flasks at 30 °C on a medium containing 1 % yeast extract and 0.11 M sodium lactate at an initial pH of 7.0. When the absorbance at 620 nm reached 0.8, the cells were harvested by centrifugation for 1 h at $2000 \times g$ and 0 °C. In most cases the cells were then washed twice by suspension in 40 ml water and centrifuging for 10 min at $5000 \times g$. Cells from two 2-l cultures were then suspended in 80 ml water and vigorously stirred with a magnetic stirrer in a 300-ml Erlenmeyer for 30 min at room temperature, in order to oxidize endogenous substrate. After centrifugation at $5000 \times g$ for 10 min, the cells were suspended in 6 ml water. This concentrated suspension could be stored at −16 °C for several months without a detectable loss of oxidative capacity.

The oxidative capacity was measured as the rate of oxygen consumption (μM O_2/min) at 25 °C in the standard medium with 0.5 mM ethanol as substrate, and related to the absorbance at 620 nm (1 cm light path). Normal values were 45–60 μM $O_2 \cdot min^{-1} \cdot A_{620\ nm}{}^{-1}$. The K_m for ethanol was 0.7 mM and that for oxygen less than 0.5 μM under these circumstances (ref. 8, cf. ref. 9).

Anaerobiosis in spectrophotometer cuvettes was attained by addition of 10 μl of a concentrated cell suspension and a small volume of ethanol, minimally 20 mM. Measurements of cytochrome redox states were not disturbed by presence of *Acetobacter* cells ($A_{620\ nm}$ about 0.5); a small signal at 445/455 nm appeared immediately at the onset of anaerobiosis, but this could easily be corrected for. Additions of inhibitors or other chemicals had no effect on the absorbance of the *Acetobacter* cells. Particles were prepared by sonication of a concentrated cell suspension.

All chemicals were of analytical grade, except those used for the growth of bacteria. Adenine nucleotides, rotenone and enzymes were from Boehringer, Mannheim. Antimycin was from Sigma, atractyloside was a kind gift of Professor V. Sprio, CCCP of Dr P. G. Heytler, and oligomycin of the Upjohn Company. Yeast extract was from Difco; the other chemicals from British Drug Houses.

Conditions for establishing reverse electron flow in the respiratory chain

The redox reaction. The ΔE per phosphorylation site should be minimal. Table I shows the calculated values of ΔE_0 for Reaction 2 with different redox couples, the ΔE_0 per phosphorylation site, and the ΔE per site when the redox couple is largely oxidized and $[O_2] = 10^{-7}$ M, which is about the maximal resolution of a normal Clark electrode.

The couples ferricyanide/ferrocyanide, oxaloacetate/malate and 2-oxoglutarate $+NH_3$/glutamate show the lowest ΔE per site at low O_2 tension.

The phosphorylation reaction. Since $-\Delta G_0$ of the ATPase reaction is maximal at $[Mg^{2+}] = 0$, a reaction medium without Mg^{2+} would be optimal. However, when

TABLE I

POTENTIAL JUMPS OVER THE RESPIRATORY CHAIN WITH DIFFERENT REDOX COUPLES

pH 7.6, 25 °C, $I = 0$.

Ref.	Redox couple	E_0 (mV)	ΔE_0* (mV)	n**	$\Delta E_0/n$ (mV)	$\Delta E/n$*** (mV)
19	Dehydroascorbate/ ascorbate	15	760	1	760	671
20	Ferricyanide/ ferrocyanide	370	405	1	405	287
21	Fumarate/succinate	−11	786	2	393	348
22	Dihydroxyacetone phosphate/glycerol 1-phosphate	−227	1002	2	501	456
23	Acetoacetate/ 3-hydroxybutyrate	−325	1100	3	367	337
22	Oxaloacetate/malate	−201	976	3	325	295
24	2-Oxoglutarate + NH_3/glutamate§	−175	950	3	317	317

* For the reaction with the O_2/H_2O couple.
** As given in Eqn 1.
*** When $[O_2] = 10^{-7}$ M and $[S]/[SH_2] = 10$.
§ $[NH_4^+] = 10^{-2}$M.

one wants to follow a reaction at low O_2 tensions for more than a few seconds, the phosphate potential is greatly lowered by the mitochondrial ATPase (see, for example, Wikström and Saris [10]). In order to keep the ATP/ADP ratio high, phosphoenolpyruvate and pyruvate kinase (EC 2.7.1.40) were added. (This means that the phosphate potential is kept high, but not constant, since the increase of P_i by action of the ATPase is not affected by the pyruvate kinase reaction.) Mg^{2+} has to be added for the pyruvate kinase reaction, which will lower the $-\Delta G_0$ of the ATPase reaction by up to 5.5 kJ [2].

We have not measured the actual phosphate potential in our experiments. One can, however, make an estimate. Since the mitochondrial ATPase activity, under the experimental conditions, was 70–170 nmol/min, the maximal rephosphorylation rate of ADP with 0.05 mg pyruvate kinase activity (13 μmol/min at infinite ADP concentration) should also be 70–170 nmol/min. The corresponding ADP concentrations calculated from these rates and the K_m for ADP (0.21 mM, see also ref. 11) are 1.3–3.2 μM. The P_i concentration, after addition of ATP, was 0.7 mM, increasing at a rate of about 70 μM/min. Thus, the phosphate potential shortly after addition of ATP would have been 66–68 kJ, decreasing at a rate of 0.3 kJ/min.

RESULTS

When rat-liver mitochondria are incubated in a medium containing substrates with a low $\Delta E_0/n$ for the redox reaction, they respire with a constant velocity of

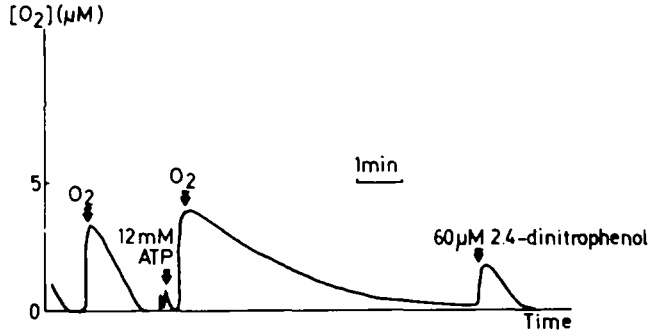

Fig. 1. Influence of phosphate potential on the mitochondrial affinity for O_2. The experiment was carried out in an Oxygraph. Standard medium plus 5 mM 2-oxoglutarate, 5 mM NH_4Cl, 1 mM sodium malonate, 5 mM phosphoenolpyruvate, 2 mM sodium arsenite, 0.05 mg pyruvate kinase. The medium was flushed with nitrogen before addition of mitochondria and pyruvate kinase. Temperature, 25 °C. Mitochondrial protein, 2.2 mg. Volume, 1.6 ml.

about 1 nmol O_2/min per mg protein, until the oxygen tension is less than 0.1 μM (Fig. 1). When the phosphate potential is raised the respiration continues, but with a strongly lowered affinity for O_2. The oxygen tension at which the velocity is half-maximal (K_m) is now measurable and is about 2–3 μM (Fig. 2), which is at least 20-fold higher than at low phosphate potential. However, in the presence of succinate, with a high $\Delta E_0/n$ for the reaction with oxygen, this increase of K_m is not seen (Fig. 3). Thus it appears that both a low $\Delta E/n$ and a high phosphate potential are necessary to cause the lowered oxygen affinity.

The effect on the K_m for oxygen is independent of the substrate couple, provi-

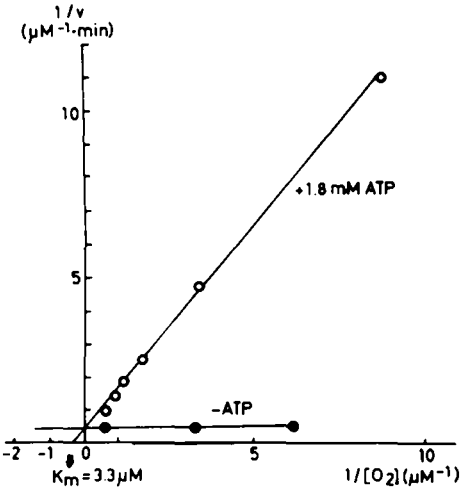

Fig. 2. Double-reciprocal plot of mitochondrial respiration at high and low phosphate potential. The experiment was carried out as in Fig. 1. Standard medium plus 5 mM oxaloacetate, 2 mM sodium arsenite, 5 mM phosphoenolpyruvate, 0.05 mg pyruvate kinase. Temperature, 25 °C. Mitochondrial protein, 3.4 mg. Volume, 1.6 ml.

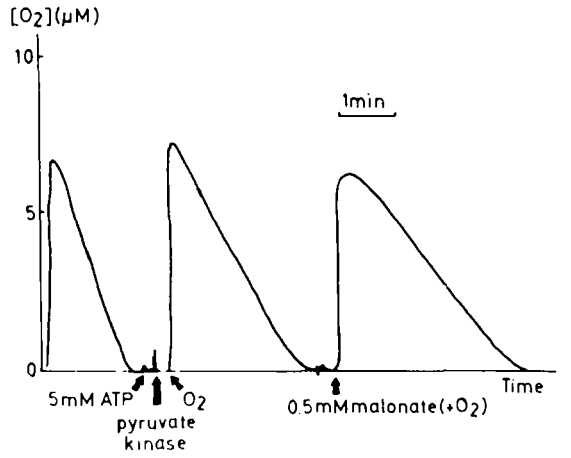

Fig. 3. Influence of phosphate potential on the affinity for O_2 of mitochondria with high $\Delta E/n$ for the redox reaction. The experiment was carried out as in Fig. 1. Medium as in Fig. 1 plus 0.3 mM succinate. Temperature, 25 °C. Mitochondrial protein, 2.7 mg. Volume, 1.6 ml.

ded that $\Delta E/n$ (Table I) is not very different. The same results were found with 2-oxoglutarate+NH_4^+, oxaloacetate and ferricyanide. Thus a direct effect of the substrate couple on cytochrome oxidase may be excluded. As is to be expected from Table I, fumarate (added as malate+rotenone) is not as effective as the above substances.

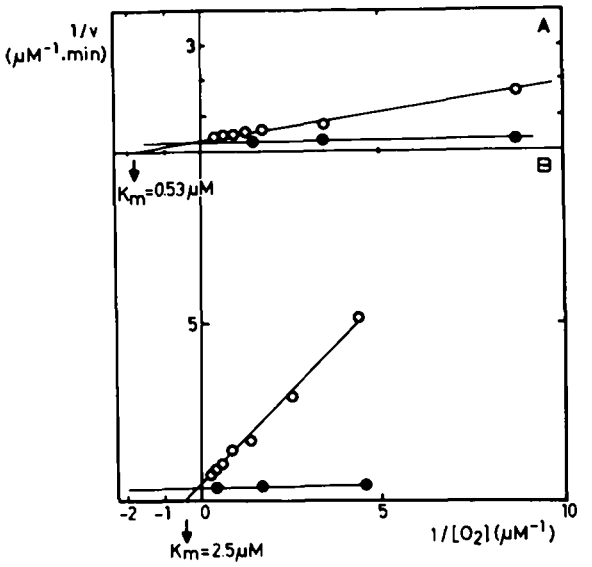

Fig. 4. Effect of low concentrations of ADP on the mitochondrial O_2 affinity, when (A) ADP is not phosphorylated, and (B) ADP is continuously phosphorylated. The experiments were carried out as in Fig. 1. A: Standard medium plus 5 mM 2-oxoglutarate, 5 mM NH_4Cl, 1 mM malonate, 2 mM arsenite. B: Medium as in A plus 5 mM phosphoenolpyruvate plus 0.05 mM mg pyruvate kinase. Temperature, 25 °C; mitochondrial protein, 3.4 mg; volume, 1.6 ml. ●, no additions; ○, plus 2 mM ATP.

TABLE II

DEPENDENCE OF THE MITOCHONDRIAL OXYGEN AFFINITY ON THE PHOSPHATE POTENTIAL

Standard medium plus 5 mM 2-oxoglutarate, 5 mM NH_4Cl, 1 mM sodium malonate, 2 mM sodium arsenite.

Additions	V (nmol O_2/min per mg protein)	K_m (μM O_2)
5 mM phosphoenolpyruvate + 0.05 mg pyruvate kinase	1.4 ± 0.5*	0.1
12 mM ATP	1.2 ± 0.4	0.45 ± 0.1
5 mM phosphoenolpyruvate + 0.05 mg pyruvate kinase + 12 mM ATP	1.6 ± 0.5	2.4 ± 0.7

* Mean ± S.D.

When no substrates are added, a small rise of K_m for oxygen can be observed on the transition from low to high phosphate potential. This can be easily explained, since in the presence of arsenite Krebs-cycle intermediates accumulate in the form of oxaloacetate and 2-oxoglutarate, and ammonia is always added with the pyruvate kinase suspension. Thus a small effect of ATP similar to that obtained on addition of oxaloacetate or 2-oxoglutarate and ammonia is to be expected.

Since ATP has a large effect only in the presence of phosphoenolpyruvate and pyruvate kinase (Fig. 4), it may be concluded that its effect on the K_m is not direct, but is due to a high [ATP]/[ADP] ratio, i.e. a high phosphate potential. A small (Table II) and transitory effect (disappearing within 2–5 min) on the K_m for O_2 can be observed when large amounts of ATP (more than 10 mM) are added without pyruvate kinase (when the phosphate potential is high just after addition of ATP but decreases rapidly by the action of the mitochondrial ATPase). Addition of 2,4-dinitrophenol, oligomycin, CCCP or atractyloside abolishes the effect of the phosphate potential on the oxygen affinity of mitochondria, bringing the K_m back to less than 0.1 μM (see, for example, Fig. 1).

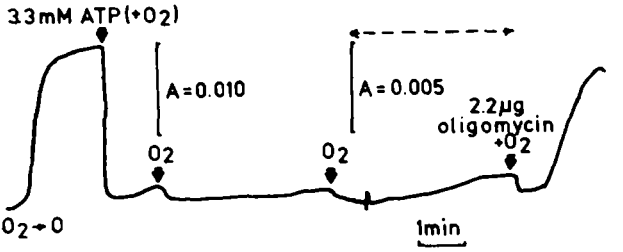

Fig. 5. Redox states of cytochrome oxidase as a function of phosphate potential measured at 445/455 nm. Standard medium plus 5 mM 2-oxoglutarate, 5 mM NH_4Cl, 1 mM malonate, 2 mM arsenite, 5 mM phosphoenolpyruvate, 0.03 mg pyruvate kinase. The cuvette was flushed with nitrogen before addition of mitochondria and pyruvate kinase. Volume 4 ml; temperature, 22 °C; mitochondrial protein, 4.4 mg.

Redox changes in the respiratory chain under conditions of high and low oxygen affinity

The cytochromes c, c_1, a and a_3 are always completely reduced under anaerobic conditions and at low phosphate potential, whatever the redox couple applied (except with ferricyanide). This is to be expected since the E_0 values of these cytochromes are higher than the highest E applied at the substrate side of the respiratory chain. When a high phosphate potential is applied, these cytochromes become oxidized (Fig. 5), as is also to be expected. Table III gives the redox states of anaerobic mitochondria under high phosphate potential with different S/SH_2 couples, compared with the affinity for oxygen (determined in separate experiments). The degree of oxidation of the cytochromes c, c_1 and aa_3 is positively correlated with the K_m for oxygen.

TABLE III

CORRELATION OF THE MITOCHONDRIAL AFFINITY FOR OXYGEN WITH THE ANAEROBIC REDUCTION LEVEL OF CYTOCHROMES $c+c_1$ AND $a+a_3$ AT HIGH AND LOW PHOSPHATE POTENTIAL

Standard medium plus 1.8 mM ATP, 2.5 mM phosphoenolpyruvate, 0.03 mg pyruvate kinase, 2 mM arsenite, 10 μl *A. mesoxydans* suspension, 0.5 μl ethanol, 4.3 mg mitochondrial protein. Volume 4.0 ml.

Additions	% reduction of cytochromes		K_m for oxygen (μM)
	$c+c_1$	$a+a_3$	
5 mM 2-oxoglutarate, 5 mM NH_4^+, 1 mM malonate	40	15	2.4
5 mM oxaloacetate, 1 mM malonate	8	12	3.3
25 mM L-malate, 2.2 μg rotenone	66	36	<0.1
5 mM glutamate, 5 mM malate, 1 mM malonate	68	56	<0.1
1 mM succinate, 2.2 μg rotenone	96	91	<0.1
None		23	0.45
5 mM 2-oxoglutarate, 5 mM NH_4^+, 1 mM malonate, 4 μM CCCP		100	<0.1

DISCUSSION

The affinity of rat-liver mitochondria for oxygen shows a clear dependence on the phosphate potential, as has also been observed by Degn and Wohlrab [12] and recently by Petersen et al. [13] (see also ref. 14). This does not, however, necessarily imply that ATP influences the K_D of oxygen and cytochrome oxidase. It is necessary to consider the kinetics of oxygen reduction before conclusions can be drawn regarding this point.

Baender and Kiese [15] reported that the rate of mitochondrial respiration is related to the oxygen concentration and the affinity according to Eqn 4.

$$v = \frac{V \cdot [O_2]^a}{K_m + [O_2]^a} \tag{4}$$

Chevillotte [16], however, pointed out that a value for a greater than 1 can be expected when cytochrome oxidase is not completely oxidized at maximal respiration rate (R_m) and when this R_m is put into the equation as V. When V is calculated as the velocity at 100 % oxidation of cytochrome aa_3, a should be 1. This then produces a straight line in the Lineweaver and Burk plot. This is supported by our data given in Figs 2 and 4, since under the conditions of our measurements cytochrome oxidase is nearly 100 % oxidized at high oxygen concentrations (Table III). Not much can be said about the slope of the line representing the kinetics in the absence of ATP, but with ATP the line is straight, as is to be expected. It is noteworthy that the effect of phosphate potential is on the K_m, with little effect on the V. The latter is affected by less than 50 %, while the former is changed by a factor of at least 20.

A complete description of the reaction in which cytochrome c is oxidized by O_2 requires a consideration of at least the following reactions (cytox represents cytochrome c oxidase)

cytox + O_2	$\rightleftharpoons$	cytox · O_2	(a)
cytox · O_2	$\rightleftharpoons$	Intermediate I	(b)
Intermediate I + c^{2+}	$\rightleftharpoons$	Intermediate II + c^{3+}	(c)
Intermediate II	$\rightleftharpoons$	Intermediate III	(d)
Intermediate III + c^{2+}	$\rightleftharpoons$	Intermediate IV + c^{3+}	(e)
Intermediate IV	$\rightleftharpoons$	Intermediate V	(f)
Intermediate V + c^{2+}	$\rightleftharpoons$	Intermediate VI + c^{3+}	(g)
Intermediate VI	$\rightleftharpoons$	Intermediate VII	(h)
Intermediate VII + c^{2+}	$\rightleftharpoons$	Intermediate VIII + c^{3+}	(i)
Intermediate VIII	$\rightleftharpoons$	cytox	(j)

in which the four protons required for the production of the two water molecules and the water molecules themselves have been omitted. At constant pH and water pressure they can be incorporated in the reaction constants.

In steady-state kinetic studies the reactions that do not involve cytochrome c or O_2 cannot be discerned, so that a simpler scheme may suffice.

$cytox^{4-} + O_2$	$\underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}}$	$cytox^{4-} \cdot O_2$	(5)
$(4\,H^+) + cytox^{4-} \cdot O_2 + c^{2+}$	$\underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}}$	$cytox^{-} + c^{3+}$ $(+2\,H_2O)$	(6)
$cytox^{-} + c^{2+}$	$\underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}}$	$cytox^{2-} + c^{3+}$	(7)
$cytox^{2-} + c^{2+}$	$\underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}}$	$cytox^{3-} + c^{3+}$	(8)
$cytox^{3-} + c^{2+}$	$\underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}}$	$cytox^{4-} + c^{3+}$	(9)
Sum:			
$(4\,H^+) + 4c^{2+} + O_2$	$\underset{k_{-6}}{\overset{k_6}{\rightleftharpoons}}$	$4c^{3+} (+2\,H_2O)$	(10)

The protons and water molecules are, rather arbitrarily, put into Eqn 6, but they may be inserted into any of the other reactions without affecting the considerations that follow.

The reaction velocity is given by

$$v = \frac{k_1 k_2 k_3 k_4 k_5(\bar{c}^{2+})^4[O_2] - k_{-1} k_{-2} k_{-3} k_{-4} k_{-5}(\bar{c}^{3+})^4}{k_1[O_2]\alpha + k_{-1}\beta + \gamma} \cdot e \tag{11}$$

where e is the total cytochrome c oxidase concentration, $\bar{c}^{2+}$ is the fraction of cytochrome c in the reduced state and $\bar{c}^{3+}$ the fraction in the oxidized state and α, β and γ are functions of $\bar{c}^{2+}$ and $\bar{c}^{3+}$ and all the reaction constants except k_1 and k_{-1} and, so far as α is concerned, except k_{-5} (see Appendix). Since the double-reciprocal plot in presence and absence of ATP shows a linear relationship between v^{-1} and $[O_2]^{-1}$, at least at O_2 concentrations above 10^{-7} M, the negative term in the numerator can be omitted in the description of the Oxygraph-visible events, so that

$$v = \frac{k_2 k_3 k_4 k_5(\bar{c}^{2+})^4 e/\alpha}{1 + \dfrac{k_{-1}\beta + \gamma}{k_1 \alpha[O_2]}} \tag{12}$$

The question to be asked is whether the known effects of ATP on the redox state of cytochrome oxidase are sufficient to explain the changes in oxygen affinity, that is, without assuming effects on Reaction 5.

It is apparent from the results that V is independent of the phosphate potential. This is to be expected since the respiration is probably caused by a stream of electrons coming from fatty acids and other endogenous substrate, or, in the case of ferricyanide clamped to cytochrome c, by the oxidation of succinate (added with malonate in order to give a respiration of about 1 nmol O_2/mg per min). In all these cases V is determined at the dehydrogenase level. At high oxygen concentration

$$v = V = \frac{k_2 k_3 k_4 k_5(\bar{c}^{2+})^4 e}{\alpha}. \tag{13}$$

Since addition of ATP to coupled mitochondria must cause a lowering of K_6 ($= k_6/k_{-6}$), one would expect a decrease of reaction constants with positive subscripts (k_{+n}) or a rise in the constants k_{-n}, or both. A lowering of the constants k_{+n} would be possible, without effect on V, if cytochrome c were correspondingly more reduced. Since c is strongly oxidized, a large relative change in $\bar{c}^{2+}$ is possible without a visible effect on the absorption. This is, however, not attractive, since the kinetics of O_2 uptake and the effect of ATP are essentially the same with ferricyanide, oxaloacetate, or ammonia–2-oxoglutarate, whereas the variability of the redox state of c would be expected to be much smaller in the presence of ferricyanide than with the two other electron acceptors. Thus, a change in the constants k_{+n} on addition of ATP is improbable, unless parallel changes occur in α, which is not an attractive assumption. An ATP-dependent effect on those k_{-n} constants that are quantitatively unimportant in α remains possible.

The K_m for oxygen, which is equal to

$$\frac{k_{-1}\beta+\gamma}{k_{-1}\alpha} \tag{14}$$

is increased by a factor of at least 20 upon addition of ATP. The question is whether the K_m can be raised so much without a change in K_1 ($= k_1/k_{-1}$). A change in α is not very likely insofar as it would imply a parallel change in the product of the k_{+n} constants as explained above. A change in γ is possible, for example by an increase in the term $k_{-2}k_{-3}k_{-4}k_{-5}(\bar{c}^{3+})^4$. Since $\bar{c}^{3+}$ remains practically constant, a change in one of the rate constants results in a corresponding change in the whole term. A change in k_{-2}, k_{-3} or k_{-4}, which appear in α, is only possible, as explained above, if the rate constant(s) concerned is (are) quantitatively unimportant in α. There is no such restriction on a change in k_{-5}, since this does not appear in α.

Thus, the observed kinetics can be explained in different ways: the simplest possibility is an effect on k_{-5}, the rate constant involving the intermediate that reacts with oxygen. The alternative possibility is a change of the dissociation constant of cytochrome oxidase and oxygen, that is in k_1 or k_{-1} or both. In all cases the limitation should be observed that

$$k_{-1}k_{-2}k_{-3}k_{-4}k_{-5}(\bar{c}^{3+})^4 < k_1k_2k_3k_4k_5(\bar{c}^{2+})^4 \cdot 10^{-4} \tag{15}$$

(where 10^{-4} atm is the p_{O_2}), in presence of ATP.

The overall equilibrium constant K_6 ($= k_6/k_{-6}$), under coupled conditions, can be calculated from the standard redox potentials of the oxygen/water and ferri-/ferrocytochrome *c* couples, and the phosphate potential. $K_6 \cdot K^2_{\text{ATPase}}$ is equal to 10^{26} (ΔG_0 of the ATPase reaction being 32 kJ, ΔE_0 for the redox couples 550 mV and taken account of 2 mol of ATP synthesized in Site 3 per mol O_2 reduced) and, with a phosphate potential of 64 kJ, $K_{6\,\text{obs}} = (k_1k_2k_3k_4k_5)/(k_{-1}k_{-2}k_{-3}k_{-4}k_{-5})$ becomes 10^{16}. This implies a $\bar{c}^{2+}$ of more than 0.001 which is not unreasonable (see Table III).

In conclusion the observed kinetics can be explained on the basis of a change in a reaction constant (k_{-5}) for the oxidation of the intermediate of cytochrome oxidase that reacts with O_2, although an effect on the reaction with oxygen cannot be excluded, especially as ATP has been reported to have an effect on the affinities of cytochrome oxidase for CO [17] and HCN [18]. However, it is questionable whether coupling in Site 3 can affect only one of the rate constants in the mechanism, since two molecules of ADP are phosphorylated for each molecule of O_2 reduced. An effect on k_{-5} alone would imply that the coupling site at Reaction 9 must provide sufficient energy for the phosphorylation of two molecules of ADP. More attractive would be a situation in which the energy is released in two steps. Before more can be said on this point, it is necessary to obtain more information on K_1, especially k_{-1}, the reaction constant that most probably limits the reverse reaction of Site 3.

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APPENDIX

The terms, α, β and γ, used in the discussion, are defined as follows:

$$\alpha = (k_3k_4k_5+k_2k_4k_5+k_2k_3k_5+k_2k_3k_4)(\bar{c}^{2+})^3 + (k_{-2}k_4k_5+k_2k_{-3}k_5+k_2k_3k_{-4})(\bar{c}^{2+})^2(\bar{c}^{3+}) + (k_{-2}k_{-3}k_5+k_2k_{-3}k_{-4})(\bar{c}^{2+})(\bar{c}^{3+})^2+k_{-2}k_{-3}k_{-4}(\bar{c}^{3+})^3.$$

$$\beta = k_3k_4k_5(\bar{c}^{2+})^3+(k_{-2}k_4k_5+k_3k_4k_{-5})(\bar{c}^{2+})^2(\bar{c}^{3+}) + (k_{-2}k_{-3}k_5+k_3k_{-4}k_{-5}+k_{-2}k_4k_{-5})(\bar{c}^{2+})(\bar{c}^{3+})^2 + (k_{-2}k_{-3}k_{-4}+k_{-3}k_{-4}k_{-5}+k_{-2}k_{-4}k_{-5}+k_{-2}k_{-3}k_{-5})(\bar{c}^{3+})^3.$$

$$\gamma = k_2k_3k_4k_5(\bar{c}^{2+})^4+k_2k_3k_4k_{-5}(\bar{c}^{2+})^3(\bar{c}^{3+})+k_2k_3k_{-4}k_{-5}(\bar{c}^{2+})^2(\bar{c}^{3+})^2 + k_2k_{-3}k_{-4}k_{-5}(\bar{c}^{2+})(\bar{c}^{3+})^3+k_{-2}k_{-3}k_{-4}k_{-5}(\bar{c}^{3+})^4.$$

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