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Effect of pH on cytochromes *b* in ATP–Mg submitochondrial particles

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SUMMARY

Addition of ATP to anaerobic, succinate-reduced phosphorylating submitochondrial particles (ATP–Mg particles) causes reduction of cytochromes *b* absorbing at 558 and 566 nm in the pH range 5.5–9.0. The extent of the reduction of both cytochromes induced by ATP is maximal at pH 7.4–7.5. On the other hand, addition of ATP to anaerobic, NADH-reduced particles causes oxidation of b_{562} at high pH, while it causes reduction of cytochromes absorbing at 558 and 566 nm at low pH. The optimal pH for the oxidation of cytochromes *b* is in the region 8.5–9.0. Partial reduction of the cytochromes absorbing at 558 and 566 nm can be brought about non-energetically by lowering the potential of the substrate redox couple or by making the reaction mixture alkaline. Addition of the electron-transfer mediator, phenazine methosulphate, to anaerobic, NADH-reduced particles causes complete reduction of cytochromes *b* absorbing at 558 and 566 nm in the pH range 5.5–9.0. The findings are interpreted in terms of a pH-induced removal of an accessibility barrier (structural or kinetic) that interferes with the redox equilibrium between NADH and cytochrome *b*.

The redox potential of cytochrome *b* in heart-muscle particles declines by 59 mV for an increase of pH by 1 unit in the pH range 6.4 (ref. 1) or 6.8 (ref. 2) to 8.2 (ref. 1). Below pH 6.8 E'_0 is independent of pH[2]. The relation between pH and cytochrome *b* in phosphorylating submitochondrial particles is of special interest in view of the fact that energy-dependent forms of cytochrome *b* with an elevated apparent midpoint potential[3–5], spectrophotometrically distinct from the conventional cytochrome *b*[6–8], have been observed in phosphorylating mitochondria and submitochondrial particles. The present report describes the effect of pH on cytochromes *b* in ATP–Mg particles both in the energized and non-energized state. A preliminary account of some of the results have been published[9].

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ATP–Mg particles were derived from heavy beef-heart mitochondria according to the method of Löw and Vallin[10]. The redox state of cytochrome *b* was followed with an Aminco-Chance dual-wavelength spectrophotometer. Tris–acetate buffers of pH 5.5–9.0 of the same ionic strength (0.05) were used.

Fig. 1 illustrates the effect of pH on the absorbance change at 566 nm in the anaerobic and aerobic steady state of ATP–Mg particles in the presence of succinate and NADH. The total absorbance change observed in the anaerobic, uncoupled state increases with increase of pH. The increase between pH 5.5 and 9.0 is approximately 2-fold in succinate-reduced particles and 1.2-fold in NADH-reduced particles. With succinate as substrate, the total absorbance change obtained in the energized state is greater than that in the non-energized, throughout the pH range studied (Fig. 1A). With NADH as substrate, however, energization brings about an increase at pH 7.0 and lower, and a decrease at pH 7.5 and higher (see Fig. 1B).

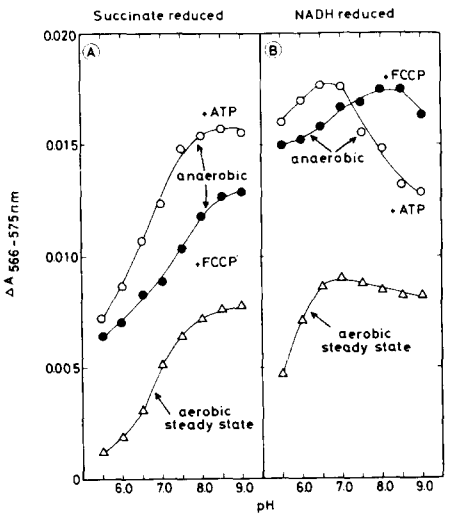

Fig. 1. Effect of pH on the absorbance at 566 nm (with reference wavelength at 575 nm) after addition of 4.2 mM succinate (A) or 1.25 mM NADH (B) to ATP–Mg particles. The particles were suspended in 200 mM sucrose, 50 mM Tris–acetate buffer (pH 5.5–9.0) and 7.5 mM $MgCl_2$ at a final concentration of 1.18 mg protein/ml. FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

The total absorbance change observed in the anaerobic, uncoupled state is greater with NADH than with succinate at all pH values studied. The difference spectrum of NADH reduced *minus* succinate-reduced particles under these conditions (not shown in this report; see ref. 7) shows absorption maxima at 558 and 566 nm, indicating that partial reduction of these cytochromes★ can be brought about non-energetically by lowering the potential of

★It is not certain whether the maximum (or shoulder) at 558 nm and that at 566 nm belong to a single cytochrome species, as proposed by Sato *et al.*[11] or to two *b* species. The variations in the relative intensities of the two bands, observed both by ourselves[7] and Wikström[12], favour but do not prove the latter possibility. In this paper, the effects at the two wavelengths will be tentatively ascribed to effects on two *b* species, indicated as b_{558} and b_{566}, respectively.

the substrate redox couple, as shown also by Wikström[12].

The pH curve obtained in the aerobic steady state parallels that in the anaerobic, energized state, which is to be expected if energy conserved during the aerobic oxidation of substrate exerts the same effect as exogenously added ATP.

In accord with the observations in Fig. 1, the difference spectrum of cytochrome *b* in anaerobic, energized, succinate-reduced particles *minus* anaerobic, uncoupled (Fig. 2A)

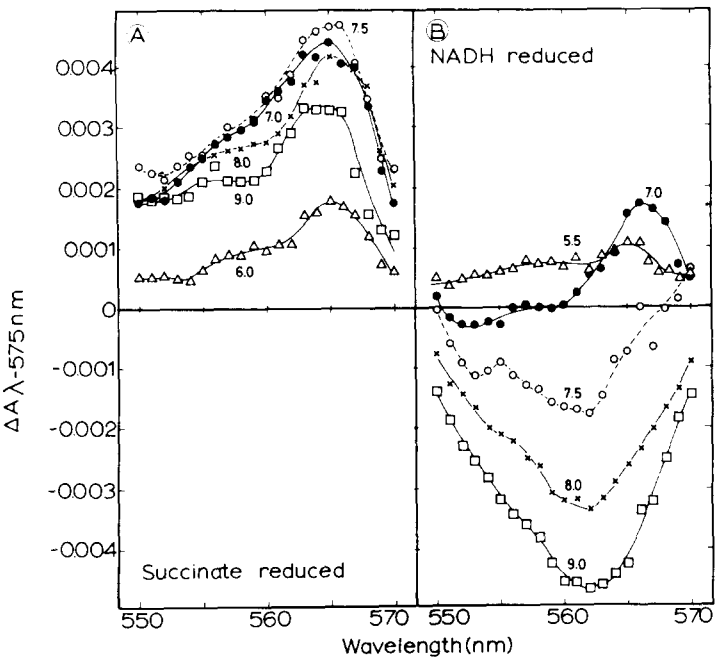

Fig. 2. Effect of pH on the difference spectrum of cytochromes *b* reduced in energized (with 1.3 mM ATP) *minus* uncoupled (with 2.1 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, FCCP) ATP–Mg particles. A. Anaerobic, succinate-reduced. B. Anaerobic, NADH-reduced. Experimental conditions are similar to those described in Fig. 1. 1.25 mg protein/ml.

clearly shows that, throughout the pH range studied, cytochromes absorbing at 558 nm (b_{558}) and 566 nm (b_{566}) are both reduced by ATP, the optimal pH for this effect being at 7.5. On the other hand, the difference spectrum in NADH-reduced particles obtained under identical condition (Fig. 2B) shows that at low pH b_{558} and b_{566} are both reduced by ATP, whereas at high pH the spectrum is dominated by the oxidation of b_{562}. At pH 7 the difference spectrum reveals an ATP-induced reduction of b_{558} and b_{566} and oxidation of b_{562}. It is noteworthy that the ATP-induced oxidation of cytochrome *b* is highest at pH 8.5–9.0, which is considerably higher than the optimal pH for the ATP-induced reduction of b_{558} and b_{566}, as shown in Fig. 2A.

Close examination of the spectra in Figs. 2A and 2B reveals that ATP causes a

significant absorbance change in the region of 550–560 nm that appears to parallel the redox changes of b_{558} and b_{566}. Preliminary EPR measurements at 83°K suggest that the unidentified component associated with the absorbance change in this region is a free-radical species with a midpoint potential about 20 mV higher than the high-potential iron–sulphur species identified by Wilson *et al.*[13]. It should be mentioned at this point that addition of the electron-transfer mediator, phenazine methosulphate, removes the absorbance change attributable to the unidentified component from the spectra of Figs. 2A and 2B.

Figs. 3A and 3B illustrate the effect of pH changes above and below neutrality on the spectrum of the cytochromes *b* in ATP–Mg particles reduced with succinate and NADH respectively. Comparison of Figs. 3A and 3B indicates that, in uncoupled, anaerobic particles, reduced by either succinate or NADH, an increase of pH above 7.0 causes reduction of b_{558} and b_{566} as already reported for rat-liver mitochondria by Azzi[14,15], whereas a decrease of pH below 7.0 causes oxidation of b_{562}. The absorbance change associated with the unidentified component in the region of 550–556 nm shows also a pH dependency at higher pH. Since above pH 6.8 the $\Delta E/\Delta$pH of b_{562} and succinate is the same (−60 mV; refs. 2 and 16) no effect of pH on the redox state of b_{562} would be expected at high pH. On the other hand, below pH 6.8, the E_0' of b_{562} does not change whereas that of succinate increases 60 mV, for every decline of 1 pH unit, so that oxidation of b_{562} would be expected below pH 7. If one applies the same argument to b_{558} and b_{566} and assumes dissociation of H^+ at a group closely linked to haem (see ref. 2), the simplest explanation for the greater reduction of b_{558} and b_{566} at high pH is that the acid pK of these cytochromes is higher than that of b_{562}. However, this explanation fails to explain that the same result is found in NADH-reduced particles, because at all pH values studied the potential of the NADH–NAD^+ system is sufficiently low to reduce b_{558} and b_{566} (E_0' −55 mV at pH 7, ref. 3) completely. This suggests that there must be an accessibility barrier (either structural or kinetic) in the segment of the respiratory chain between NADH dehydrogenase and cytochrome *b* that interferes with the redox equilibrium between NADH and cytochromes *b*. It is likely that the effect of high pH on b_{558} and b_{566} in NADH reduced particles is directly associated with removal of the barrier. This explanation is supported by the experiment shown in Fig. 3C. When the cytochromes *b* are reduced by NADH in the presence of the electron-transfer mediator phenazine methosulphate (*cf.* Fig. 4, Trace A), no effect of pH change is observed in the uncoupled state throughout the pH range studied. Phenazine methosulphate removes the accessibility barrier to NADH.

In contrast to this result with NADH, addition of phenazine methosulphate does not cause any further reduction in anaerobic, succinate-reduced particles (Fig. 4, Trace C). Since the potential of the reaction mixture under these particular conditions (+35 mV, kindly measured by Mr. F.R. Opperdoes using the procedure described in ref. 5) is too high to reduce b_{558} and b_{566} (E_0' −55mV), no effect of phenazine methosulphate on reduction of cytochrome *b* by succinate is to be expected. The effect of ATP on b_{558} and b_{566} was the same in the presence and absence of phenazine methosulphate throughout the pH range studied.

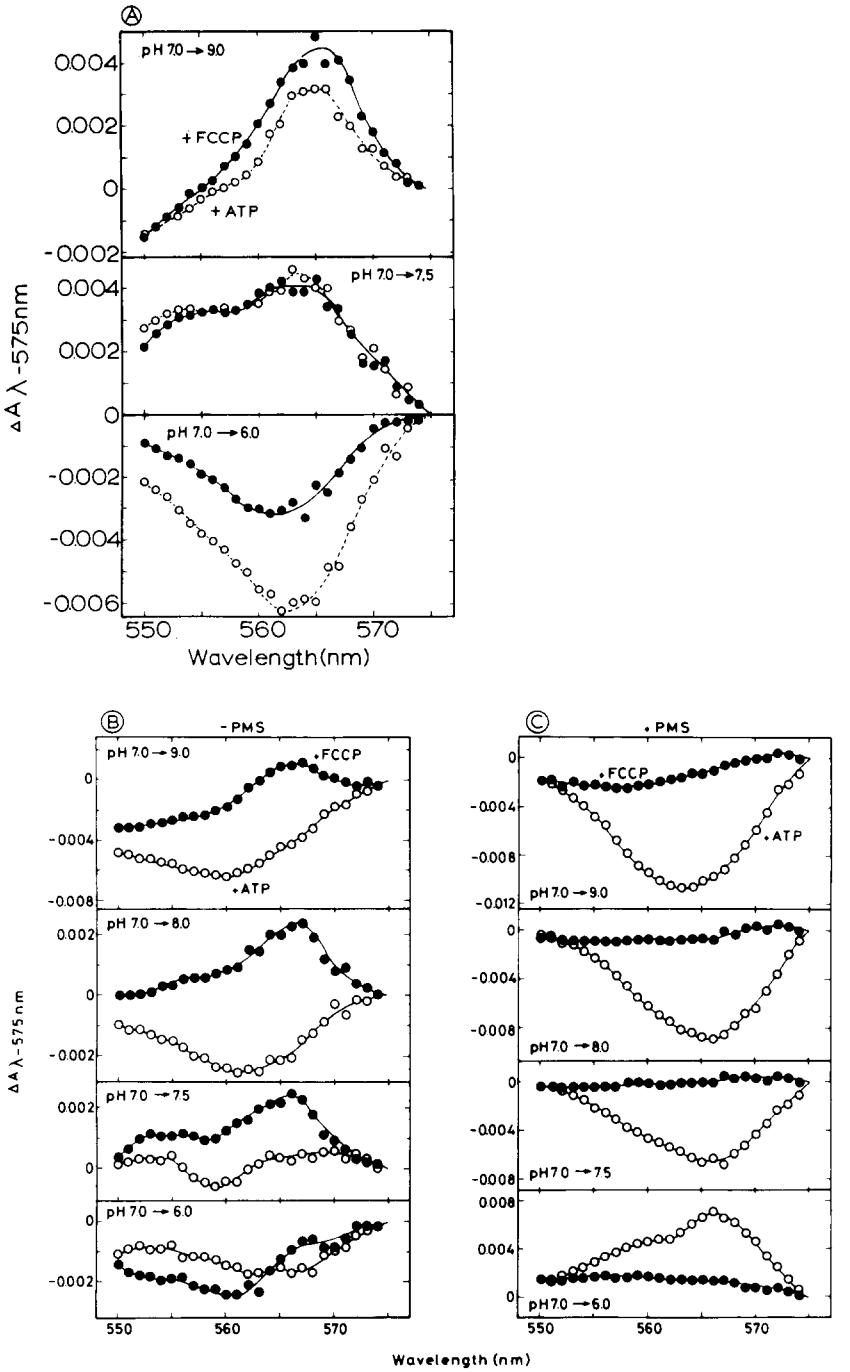

Fig. 3. Effect of pH changes on the redox level of cytochromes *b* in energized (○——○) and uncoupled (●——●) ATP–Mg particles. pH 7.0 was used as a reference. A. Anaerobic, succinate-reduced in the absence of phenazine methosulphate. Essentially the same curves were obtained in the presence of phenazine methosulphate. B. Anaerobic, NADH-reduced in the absence of phenazine methosulphate (PMS). C. Anaerobic, NADH-reduced particles in the presence of 20 μM phenazine methosulphate. The data of A and B refer to the same experiment shown in Fig. 2. The experimental conditions of C were identical except that phenazine methosulphate (20 μM) was added after anaerobiosis.
1.67 mg protein/ml.

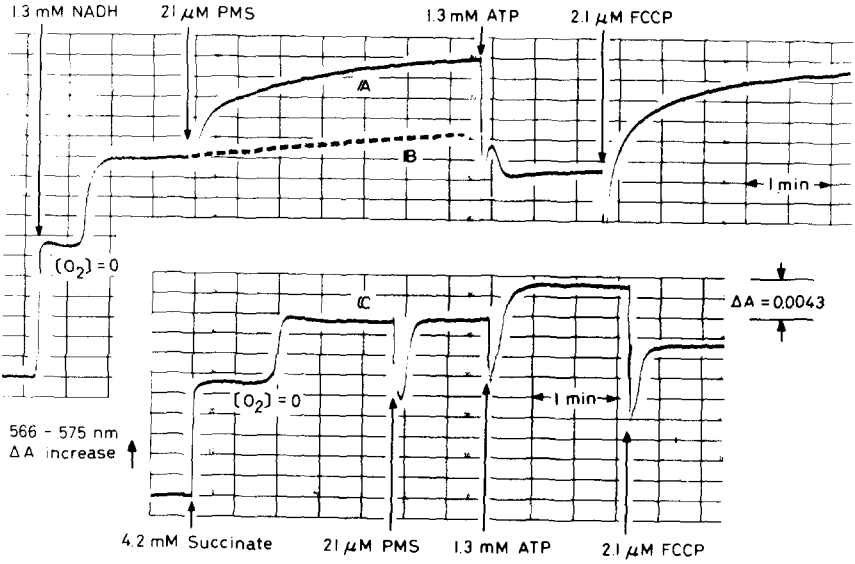

Fig. 4. Comparison of effect of phenazine methosulphate on the redox state of cytochrome *b* in ATP–Mg particles reduced with NADH (Trace A) and with succinate (Trace C). Trace B (dotted line) shows the absorbance increase in NADH-reduced particles after anaerobiosis in the absence of phenazine methosulphate. Experimental conditions are similar to those described in Fig. 1. pH of the reaction mixture, 7.5; protein concentration, 1.67 mg/ml.

In the presence of NADH, phenazine methosulphate and ATP, raising the pH results in the oxidation of b_{562}, which is to be expected since the ATP-induced oxidation of *b*, due to reversal of Site I, is optimal at pH 8.5–9.0 (Fig. 2B). Lowering the pH from 7.0 to 6.0 causes reduction of b_{558} and b_{562} to a level slightly above that obtained in the presence of uncoupler. At pH 6.0, then, ATP specifically induces the reduction of these cytochromes even in the presence of phenazine methosulphate. It is not possible to say whether this also occurs at higher pH, since the oxidation of b_{562} then dominates the difference spectra. Lowering of the pH brings about a greater oxidation of b_{562} in the presence of ATP than in the presence of uncoupler (Fig. 3A). Since the redox potential of b_{562} is scarcely changed in this pH region, it is probable that the E'_0 of the 'electron sink' to which electrons disappear under the influence of energy[7], under these conditions, is raised by lowering the pH.

The nature of the accessibility barrier in the respiratory chain that interferes with the redox equilibrium between NADH and b_{558} and b_{566} is not known. Electrons derived from NADH can slowly penetrate the barrier in the absence of phenazine methosulphate (Fig. 4, Trace B) and more rapidly and completely in the presence of phenazine methosulphate (Trace C). Experiments to be described elsewhere show that addition of 3–5 mM cyanide either before the addition of NADH or after anaerobiosis inhibits the slow reduction shown in Trace B. In this respect the barrier has characteristics proposed for Component X by Baum *et al.*[17].

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