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Effect of ^2H_2O on energy-dependent oxidoreduction of cytochrome *b*

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SUMMARY

The rate of oxidation of the long-wavelength cytochrome *b* (b_{566}) on addition of oligomycin to ATP-supplemented anaerobic rat-liver mitochondria was strongly inhibited when ^2H_2O was substituted for medium water. This effect was dependent on added substrate, was reversed by uncoupling agents, and was absent in sub-mitochondrial particles. At the same phosphate potential, b_{566} reduction was favoured in ^2H_2O in comparison with H_2O medium. The redox state of b_{566} may be controlled by the phosphate potential via an intramembrane acid–base equilibrium which is shifted by ^2H_2O.

The discovery of an energy-linked change in the midpoint potential of the long-wavelength cytochrome *b* (refs. 1–3) led to the proposal[2] of an energy-linked chemical modification of this species (cytochrome b_T, ref. 2) to a 'high-energy' form ($b_T \sim I$) with a midpoint potential almost 300 mV higher than that of the 'low-energy' form (b_T). The absorption spectra of the reduced low and high potential forms are identical[3–5] (but see refs. 6–8). Thus the evidence for an energy-linked chemical modification rests on the potentiometrical data. It is, however, surprising that a change in midpoint potential of nearly 300 mV, if it is due to a chemical (or conformational) modification of the haem as proposed by Chance *et al.*[2], does not cause a shift in the absorption spectrum (contrast refs. 6–8). Although such a change may be possible, other explanations, not expected to involve spectral alterations, should seriously be considered. One possibility of this kind is a dependence of the midpoint potential on the proton activity in the vicinity of the cytochrome molecule[5], and on the pK_a values[15] of acidic groups which may be involved in the oxidoreduction mechanism[9–12]. Both the proton activity[13,14] and the pK_a values[15] in the membrane phase may vary considerably with the 'energy state' of the mitochondrion.

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The redox state of the long-wavelength cytochrome *b* (b_{566}, refs. 4, 5, 16) is dependent on the phosphate potential[1–8,16]. As seen in Fig. 1 the same concentration of ATP, added several times sequentially, caused less reduction of b_{566} after each addition due to the increasing concentration of ADP + P_i. In ^2H_2O medium, however, the maximal extent of reduction decreased very little after the same decrease in phosphate potential. The experiment is continued in Fig. 2 where it is seen that the fourth ATP addition caused a very small cycle in H_2O medium, while the corresponding response in ^2H_2O was almost as large as after the first ATP addition. Less ATP was also required for complete reduction in ^2H_2O in comparison with H_2O medium.

Addition of oligomycin (Fig. 2) results in spontaneous discharge of the 'energized' state with oxidation of b_{566}. The rate of oxidation was considerably slower and had a longer lag-time in ^2H_2O. 75–90% inhibition of the maximal rate was observed in 94–96% ^2H_2O, as compared with the rate in H_2O medium. If the discharge was induced by an uncoupling agent rather than by oligomycin[5], b_{566} was rapidly oxidized in both ^2H_2O and H_2O without a significant difference in rates. Similar results were obtained with cyanide-supplemented mitochondria rather than anaerobic[5]. Added substrate (succinate or *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) + ascorbate[5]) was essential, since in the presence of only endogenous substrates, cyanide and ATP, reoxidation of b_{566} after the oligomycin addition was almost unaffected by ^2H_2O substitution. In the absence of added substrates, but not in their presence, ATP caused oxidation of cytochromes $c + c_1$ and *a* in addition to the reduction of b_{566}.

The rate of endogenous dissipation of energy is high in sub-mitochondrial particles in contrast to intact mitochondria as revealed *e.g.* by high Mg^{2+}-stimulated ATPase activity and rapid oxidation of b_{566} after addition of oligomycin (Fig. 3). ^2H_2O had no appreciable

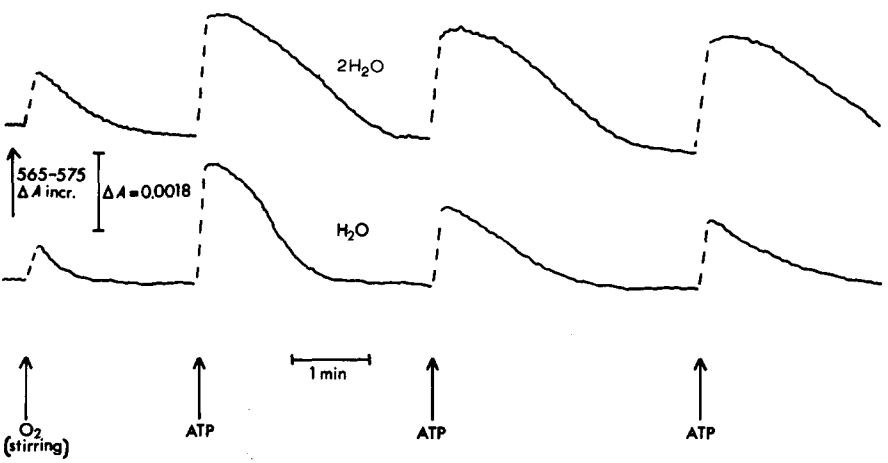

Fig. 1. Effect of deuterium oxide on the relation between redox state of b_{566} and phosphate potential. 0.2 M mannitol–0.07 M sucrose–0.02 M KCl–0.03 M Tris–HCl was suspended in either ^2H_2O (p^2H 7.4) or H_2O (pH 7.4). To this medium was added 5 μM rotenone, 6.7 mM Tris–succinate and rat-liver mitochondria (1.8 mg protein/ml). In the ^2H_2O experiments the final ^2H_2O content was 94–96%. After anaerobiosis the mitochondria were incubated for 3 min, after which ATP additions of 17 μM each were made as shown in the figure. An Aminco–Chance dual-wavelength spectrophotometer (light path 1 cm, slit width 0.2 mm) was used for all measurements reported.

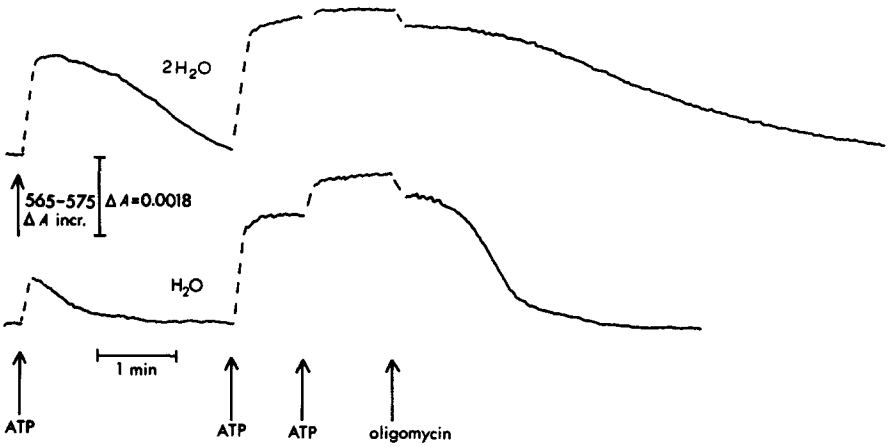

Fig. 2. Effect of deuterium oxide on the relation between redox state of b_{566} and phosphate potential. The figure shows the continuation of the experiment of Fig. 1. The additions shown are (in the order of addition): 17 μM ATP, 170 μM ATP, 1.3 mM ATP and 5 μg/ml oligomycin.

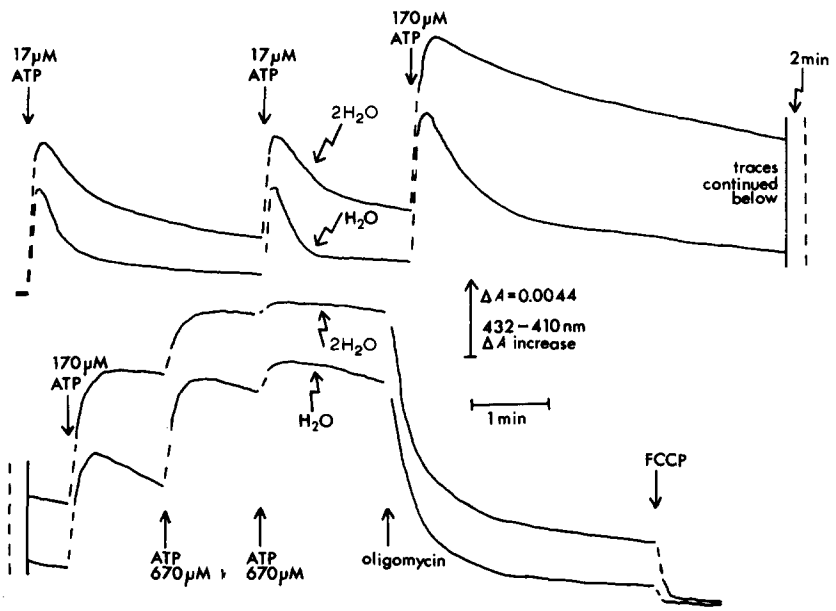

Fig. 3. Effect of deuterium oxide on the relation between redox state of b_{566} and phosphate potential in Mg^{2+}-ATP particles[17]. To the standard medium (see legend to Fig. 1) was added 5 μM rotenone, particles (1 mg protein/ml), 3.3 mM KCN and 6.7 mM Tris–succinate. The suspension was incubated for 8 min, after which the following additions were made (in the order of addition): Two times 17 μM ATP, 170 μM ATP (upper part of the figure), 170 μM ATP, 2 times 670 μM ATP, 3 μg/ml oligomycin and 1 μM carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) (lower part).

effect on the rate of this reaction in particles (contrast mitochondria, Fig. 2). At very high or very low phosphate potentials the rate of b_{566} oxidation after an ATP pulse (Fig. 3) was only slightly inhibited by ^2H_2O, the $k_{H_2O}/k^2_{H_2O}$ ratio being approx. 2 (pseudo-first-order rate constants). At intermediate phosphate potentials (third ATP addition in Fig. 3) $k_{H_2O}/k^2_{H_2O}$ was above 6 for the initial part of the reaction, but only 2 for the slow part where the reaction had nearly reached a stationary state. In this state, where b_{566} is presumably in equilibrium with a nearly constant 'energy pressure', the concentration of reduced b_{566} was considerably higher in ^2H_2O than in H_2O. This difference was pronounced at intermediate phosphate potentials, but was much less at low or high (saturating) potentials.

These data (Figs. 1–3) suggest that an equilibrium between the 'energy pressure' and b_{566} may be shifted by ^2H_2O so that a lower 'energy pressure' is required for 50% reduction in ^2H_2O than in H_2O medium. According to this explanation the rate of b_{566} oxidation would be inhibited by ^2H_2O only when this reaction proceeds with the redox state of b_{566} in equilibrium with the 'energy pressure'. This would be the case only when the net rate of energy dissipation $\left(-\frac{d(\sim)}{dt}\right)$; $(\sim)$ denoting the magnitude of the 'energy pressure') is low in comparison with the rate of b_{566} reduction (the back-reaction). $-\frac{d(\sim)}{dt}$ is high after addition of an uncoupler, or addition of oligomycin to Mg^{2+}-ATP particles (Fig. 3), and the rate of the back-reaction is low in the absence of added substrate due to a low concentration of electron donors. Therefore, under these conditions, the rate of oxidation of b_{566} is affected very little or not at all by ^2H_2O. Inhibition of the energy dissipation reaction *per se* by ^2H_2O is in agreement with some but not with all experimental results (Figs. 1–3). A shift of an equilibrium prior to the rate-determining step by ^2H_2O (see ref. 18) is, however, fully consistent with all the available data.

A shift in the equilibrium between b_{566} and the 'energy pressure' by ^2H_2O indicates that this equilibrium may be of acid–base nature, *e.g.*

$$H^+ \cdot b^{2+}_{566} + H_2O \rightleftharpoons b^{3+}_{566} + H_3O^+ + e^- \qquad (1)$$

Although cytochrome *b* may release one proton on oxidation[10–12], the proton dependence could also be indirect *e.g.* through ubiquinone. The 'energy pressure' may simply be a high proton activity in a region of the membrane of low water activity[13]. Dehydration of this region, *e.g.* by means of a primary conformational change in functional proteins[19], or directly by the reaction[13,14]

$$ATP + H_2O \rightarrow ADP + P_i \qquad (2)$$

would result in an increase in the pK_a of $H^+ \cdot b_{566}$ and in the proton activity, from which follows a higher midpoint potential, since

$$E_m = E_0 + \frac{RT}{nF} \ln \frac{(H_3O^+)}{(H_2O)K_a} \qquad (3)$$

^2H_2O is expected to decrease the K_a value[18] with an increase in the midpoint potential.

This will in turn favour reduction of b_{566} as was indeed observed. Work is in progress to elucidate the effect of ^2H_2O in more quantitative terms.

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