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WATER AND CYTOCHROME OXIDATION–REDUCTION REACTIONS

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SUMMARY

Heavy water (^2H_2O) substitution for H_2O in photosynthetic bacterial whole cells and mitochondrial membrane fragments results in an isotope effect of $\sqrt{2}$ for the ratio of the half-times [$t_{\frac{1}{2}(^2H_2O)}/t_{\frac{1}{2}(H_2O)}$] of oxidation–reduction reactions involving cytochromes. This result, along with the absolute requirement for water, and the effects on the half-times of the substitution of hydrogen-bonding molecules such as ethylene glycol and glycerol for water suggest the direct participation of hydrogen atoms of water and water rotation in the rate-determining step of these electron transfer reactions. Arrhenius plots of cytochrome photooxidation half-times measured at various temperatures from 300 to 77 °K show the half-times to be temperature-dependent from 300 °K to about 150 °K and temperature-independent from about 150 °K through liquid N_2 temperatures. ^2H_2O substitution does not change the qualitative response of half-times to temperature; however, the half-times are retarded by $\sqrt{2}$ throughout the entire temperature range investigated. If the $\sqrt{2}$ factor found in these experiments is indicative of proton or hydrogen atom motion, then direct electron transfer by electron tunnelling can not be the rate-limiting step in the cytochrome oxidation–reduction processes studied. The experimental results suggest that electron transfer between membrane-bound electron carrier molecules may be effected by means of hydrogen atom transfer *via* "water bridges" in biological oxidation–reduction reactions.

INTRODUCTION

The possible role of water in biological oxidation–reduction reactions *in vivo* can be most effectively studied with the whole cells of photosynthetic bacteria from which cellular water has been removed by lyophilization (freeze-drying). Light-induced absorbance changes may be first studied in the absence of water. The lyophilized material can then be reconstituted with H_2O or ^2H_2O or with water soluble compounds and the rates of light-induced reactions studied.

This study has shown that water is essential for cytochrome oxidation–reduction reactions in photosynthetic bacteria and that ^2H_2O substitution results in the retardation of the half-times of these reactions. The ratio of the half-times for

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the transient kinetics in ^2H_2O and H_2O reconstituted systems is close to the value of $\sqrt{2}$. The same magnitude of the deuterium isotope effect has been observed in mitochondrial as well as photosynthetic electron transfer systems. In the case of cytochrome oxidation in certain photosynthetic bacteria, the $\sqrt{2}$ isotope effect holds even at liquid N_2 temperatures.

The activation energies for cytochrome reduction have been found to be much larger than those for cytochrome oxidation in the different electron transfer systems studied.

A preliminary report on the present work was presented previously[1].

MATERIALS AND METHODS

Photosynthetic bacteria

The two species of non-sulfur photosynthetic bacteria, *Rhodopseudomonas gelatinosa* strain I and *Rhodopseudomonas* sp. NW were grown photoheterotrophically in a malate culture medium[2]. The two species of sulfur purple bacteria, *Thiocapsa floridana* and *Chromatium vinosum*, were grown in modified Pfennig's medium[3].

Whole cells were harvested after 20–48 h of growth and were resuspended in distilled water prior to lyophilization. Whole cells used for control experiments were resuspended in a small volume of supernatant culture medium to give samples with a bacteriochlorophyll concentration of about 0.3 mM.

Oxidation of cytochrome *c* was initiated, by means of a photosynthetic bacteriochlorophyll system, with a Q-switched liquid dye laser pulse having a pulse width of about 30 ns and a wavelength of either 860 or 960 nm (ref. 4). The reaction rates were measured with a single beam spectrophotometer as described previously[5,6].

In the whole cells of the non-sulfur bacteria used in our experiments a *c*-type cytochrome, identified as a "low potential" cytochrome *c* (refs 4 and 6), is oxidized. Spectrally, it can be identified on the basis of an absorption maximum at about 406 nm and a minimum at 422–423 nm. Most of the low potential cytochrome *c* of the whole cells of the sulfur bacteria would be in the oxidized form under the experimental conditions employed. Thus, in this case the main species that undergoes oxidation is a "high potential" cytochrome *c* (ref. 7). The spectral characteristics of both of these *c*-type cytochromes are similar and they are referred to as cytochrome *c* in this part of the paper.

Lyophilization of samples

Cultures of photosynthetic bacteria and other microorganisms are routinely lyophilized (freeze-dried) commercially (American Type Culure Collection, Rockville, Md.) and sealed in ampules for storage and preservation. When liquid cultures of such microorganisms are needed, sterile culture medium is added to the freeze-dried powder-like material. With the exception of chemically-bound water the lyophilization method employed effectively removes water from the bacteria and mitochondria. Under a phase contrast light microscope lyophilized whole cells can be seen to become motile, and remain so for many hours, upon addition of a drop of distilled water onto the slide indicating these cells survive lyophilization and are potentially viable and capable of resuming normal cellular activity. Other biological materials such as certain proteins are frequently freeze-dried for similar purposes.

Lyophilized mammalian cytochrome *c* (ref. 8) and bacterial cytochromes retain full enzymatic activities.

Freeze-drying of whole cells of photosynthetic bacteria was carried out for 12–20 h in a Thermovac model FD-2 freeze-dryer operated under 25 μm vacuum.

RESULTS AND DISCUSSION

Cytochrome oxidation and reduction kinetics of H_2O and ^2H_2O reconstituted systems

We have applied the technique of lyophilization to the investigations on the effect of removal and addition of water (distilled from an all-glass distillation apparatus), and of substituting water with deuterium oxide (^2H_2O, 99.81%, ICN Corp., Irvine, Calif.) on the half-times of cytochrome oxidation and reduction transient kinetics. We have also observed the effect of replacing H_2O with various mixtures of water miscible organic solvents on the half-time of cytochrome oxidation in *R. gelatinosa*.

Cytochrome oxidation can not be observed in lyophilized cells; however, absorbance changes can be seen (Fig. 1A) in dry cells which may be correlated with bacteriochlorophyll charge separation and carotenoid band shifts. As shown in Fig. 1B, the full extent of cytochrome oxidation is observed upon wetting of the sample with a small amount of water added into the sample cuvette. The reaction rates in lyophilized whole cells freshly reconstituted with H_2O do not differ significantly from those of unlyophilized whole cells used as a control. The oxidation kinetics

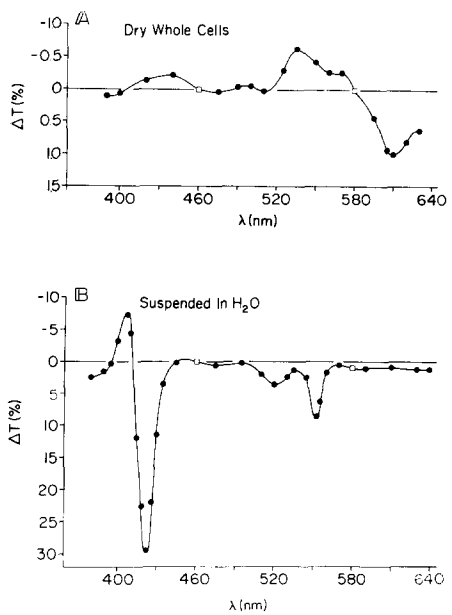

Fig. 1 (A) Light-*minus*-dark difference spectrum of freeze-dried whole cells of *R. gelatinosa* at 298 °K showing light-induced absorbance changes of bacteriochlorophyll and carotenoids. No cytochrome reactions are observed. (B) Light-*minus*-dark difference spectrum of the same dry sample reconstituted in H_2O showing the oxidation of a *c*-type cytochrome.

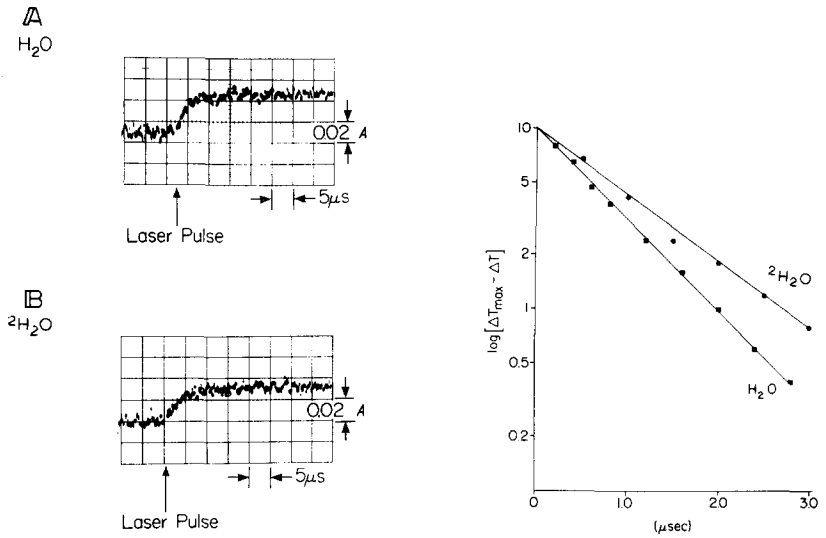

Fig. 2. Typical oscilloscope traces showing the kinetics of cytochrome *c* oxidation at 298 °K in reconstituted lyophilized whole cells of *C. vinosum* monitored at 423 nm: (A) in H_2O; (B) in ^2H_2O.

Fig. 3. The kinetics of cytochrome *c* oxidation at 298 °K in *R. gelatinosa* lyophilized whole cells reconstituted in H_2O and ^2H_2O, plotted on a semi-logarithmic graph paper. The ordinate gives the logarithm of amplitude of cytochrome oxidized in arbitrary units. Time in μs elapsed after a single laser flash is plotted on the abscissa. The traces used are those corresponding approximately to the average rates obtained for this species.

of both H_2O and $2H_2O$ samples appear to be monophasic on the time scale used (Figs 2 and 3). The cytochrome reactions were followed at the wavelength 423 nm.

We have found that by substituting H_2O with ^2H_2O in our reconstitution studies it is possible to retard consistently the rates of cytochrome oxidation and reduction reactions. The rate-retarding effect of ^2H_2O was established rapidly and did not depend on the time of incubation. Some of the results obtained for the half-times of oxidation and reduction of the *c*-type cytochromes in the photosynthetic bacteria are given in Table I. Each of the values shown is the result of averaging from 10 to 30 individual values obained from separate experiments. The ^2H_2O to H_2O ratio is the ratio of the average value of half-times for ^2H_2O to the average value of half-times for H_2O and was always obtained from measurements with samples reconstituted from the same lyophilized preparation. The uncertainties for half-times shown are standard errors. These have been propagated through to the ^2H_2O to H_2O ratio and are given as the ^2H_2O to H_2O ratio times the square root of the sum of the squares of the fractional standard errors for the ^2H_2O and H_2O half-time values.

The reduction of the *c*-type cytochrome in *R. gelatinosa* is very slow compared with the oxidation, and hence good signal-to-noise ratio can be obtained (Fig. 4). The reduction kinetics (Figs 4 and 5) are clearly not monophasic and show apparent biphasicity on the time scale used. A possible explanation is that there are two electron donors for the cytochrome. Though the electron donors for photooxidized

TABLE I

OXIDATION AND REDUCTION RATES OF *c*-TYPE CYTOCHROMES IN H_2O AND ^2H_2O

Some of the results of half-time ($t_{1/2}$) measurements for cytochrome *c* oxidation and reduction in photosynthetic bacteria are tabulated: control, unlyophilized whole cells; H_2O, lyophilized whole cells reconstituted with distilled water; ^2H_2O, lyophilized whole cells reconstituted with ^2H_2O (99.81%). H_2O and ^2H_2O samples were prepared from the same batch of lyophilized whole cells in all cases except for the 298 °K oxidation rates in *R. gelatinosa*. The half-times given in the table are average values with standard errors.

	$t_{1/2}$ (μs) *Cytochrome c oxidation*						$t_{1/2}$ (*ms*) *Cytochrome c reduction*	
	298 °K				*80 °K*		*298 °K*	
							R. gelatinosa	
	R. gelatinosa	*Rps. sp. NW*	*T. floridana*	*C. vinosum*	*R. gelatinosa*	*Rps. sp. NW*	*Fast phase*	*Slow phase*
Control	0.70 ± 0.05	0.55 ± 0.03	1.80 ± 0.12	2.50 ± 0.12	4.60 ± 0.15	4.50 ± 0.15	210 ± 10	1100 ± 50
H_2O	0.96 ± 0.05	0.53 ± 0.02	1.30 ± 0.03	2.19 ± 0.12	4.23 ± 0.20	4.32 ± 0.20	180 ± 8	1380 ± 30
^2H_2O	1.35 ± 0.03	0.70 ± 0.01	1.62 ± 0.04	3.11 ± 0.11	6.04 ± 0.44	6.06 ± 0.29	250 ± 10	1960 ± 40
$^2H_2O/H_2O$	1.40 ± 0.09	1.34 ± 0.06	1.25 ± 0.04	1.42 ± 0.10	1.43 ± 0.12	1.40 ± 0.09	1.39 ± 0.08	1.42 ± 0.04

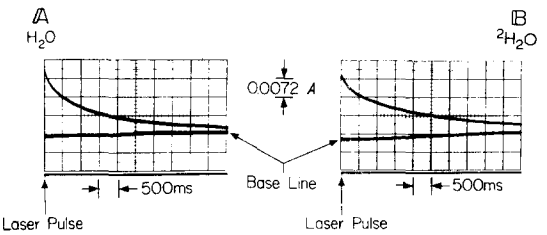

Fig. 4. The kinetics of cytochrome *c* reduction at 298 °K in reconstituted lyophilized whole cells of *R. gelatinosa* monitored at 423 nm: (A) in H_2O; (B) in ^2H_2O.

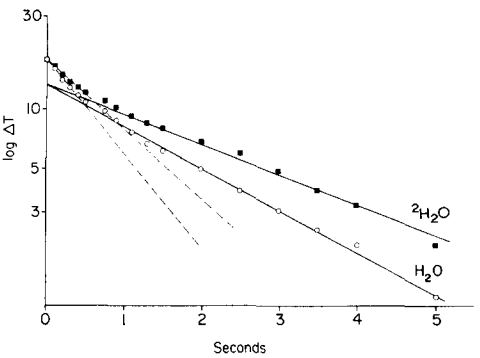

Fig. 5. Semi-logarithmic plot of apparent biphasic cytochrome *c* reduction kinetics of Fig. 4. The logarithm of reaction amplitude in arbitrary units measured as change in transmission is plotted on the ordinate. Time in seconds after a laser flash is plotted on the abscissa.

c-type cytochromes in purple bacteria are not well defined, it is possible that the reductant responsible for the fast recovery phase is within the cyclic system of photosynthetic electron transport and that the slow phase is coupled to substrate oxidation. An alternative explanation in terms of a diffusion model involving hydrogen atom displacements in a water bridge is also possible and will be discussed elsewhere. It is also possible that the slow phase may represent a conformational change subsequent to electron transfer; such biphasic results have been obtained in soluble cytochrome *c* reduction experiments by hydrated electrons which were produced by pulsed radiolysis[9]. Significant conformational differences have been found in the X-ray structures of oxidized and reduced cytochrome *c* (ref. 10).

It is evident from Fig. 5 that the rate-retarding effect of deuterium isotope substitution is observed on the rates of both phases of cytochrome reduction as well. The average half-time, obtained from many separate measurements, for the fast phase is 180 ± 8 ms and that for the slow phase is 1380 ± 30 ms in H_2O reconstituted whole cells. ^2H_2O substitution has yielded a half-time of 250 ± 10 ms for the fast phase and 1960 ± 40 ms for the slow phase (Table I). The uncertainties originating from the low level of noise present in the traces are very small. The ratio of average half-times, $t_{½(^2HO_2)}/t_{½(H_2O)}$ is 1.39 ± 0.08 for the fast phase and 1.42 ± 0.04 for the slow phase.

Reconstitution of lyophilized cells with ^2H_2O caused no significant change in the amount of cytochrome *c* oxidized. Quantum efficiency measurements of the

oxidation reaction for both H_2O and ^2H_2O samples gave an average value of 0.11 with 960-nm laser light, and values that approximate the theoretical maximum of 1.0 with 860-nm light[4]. The only observed difference between the two samples was in the half-times of oxidation and reduction.

It has also been shown that the light-induced carotenoid shift at 508 nm in *R. spheroides* and the 518-nm absorption change in *C. reinhardi*, both non-electron transfer processes, do not show a deuterium isotope effect[11].

Temperature dependence of reactions

Arrhenius plots for cytochrome oxidation half-times in *R. gelatinosa* and *Rhodopseudomonas* sp. NW whole cells measured over a temperature range from 300 to 77 °K show temperature-dependence of the reaction rates from about 150 °K through 300 °K, and apparent temperature-independence, characterized by an activation energy of approximately zero, from 150 °K through liquid N_2 temperatures as shown typically in Fig. 6. ^2H_2O substitution does not change the qualitative response of rates to temperature; however, the half-times increase by a factor of 1.3–1.4 throughout the entire temperature range. As is evident in Fig. 6 the deuterium isotope effect persists even where the rates show apparent independence of temperature. In the range where the rates show temperature dependence an average activation energy (E_a) of 2.3 ± 0.4 kcal/mole was obtained with *R. gelatinosa*, and an average of 2.1 ± 0.4 kcal/mole with *Rhodopseudomonas* sp. NW. No significant deuterium isotope effect could be observed for the activation energies of these cytochrome oxidation reactions.

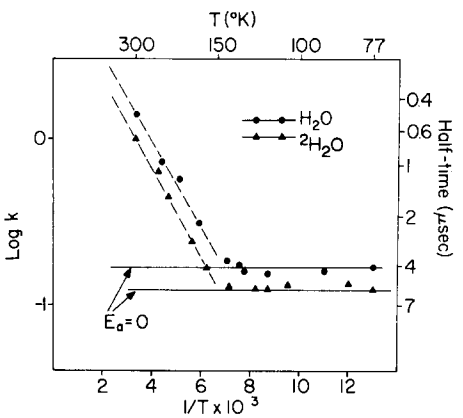

Fig. 6. Cytochrome *c* oxidation reaction rates in *Rhodopseudomonas* sp. NW lyophilized whole cells, reconstituted in H_2O and ^2H_2O, were determined at various sample temperatures. The logarithm of rate constant is plotted on the ordinate. The abscissa is proportional to the reciprocal of absolute temperature.

The activation energy for cytochrome reduction obtained from half-time measurements between 5 to 30 °C in *R. gelatinosa* has been found to be considerably greater than that for oxidation. E_a of fast phase is 11.3 ± 1.5 kcal/mole and that of the slow phase is 10.4 ± 1.8 kcal/mole. These values are in the similar range as for the reduction of the *c*-type cytochrome, C_{553} in *Chromatium* sp. D (Kihara, T., unpublished observations).

Substitution experiments

The results of ^2H_2O replacement experiments have led to additional substitution experiments using highly water miscible substances. Fig. 7 shows the effect of substituting water with aqueous (H_2O) mixtures of ethylene glycol, glycerol, and *p*-dioxane on the half-times of cytochrome oxidation. *p*-Dioxane, the least viscous, exerted the most profound inhibitory effect; at 10% (1.18 M) the reaction was completely blocked. Glycerol, the most viscous, requires a very high concentration of 90% (12.3 M) to achieve the same result; at 65% the slowest half-time of 2.5 μs was reached. Ethylene glycol, intermediate in viscosity, is most effective in increasing the half-time which goes up to 100 μs at 70% (12.6 M). At about 80% (14.4 M) the reaction becomes completely inhibited. These results indicate that the viscosity of the medium is apparently not a factor influencing the oxidation rate.

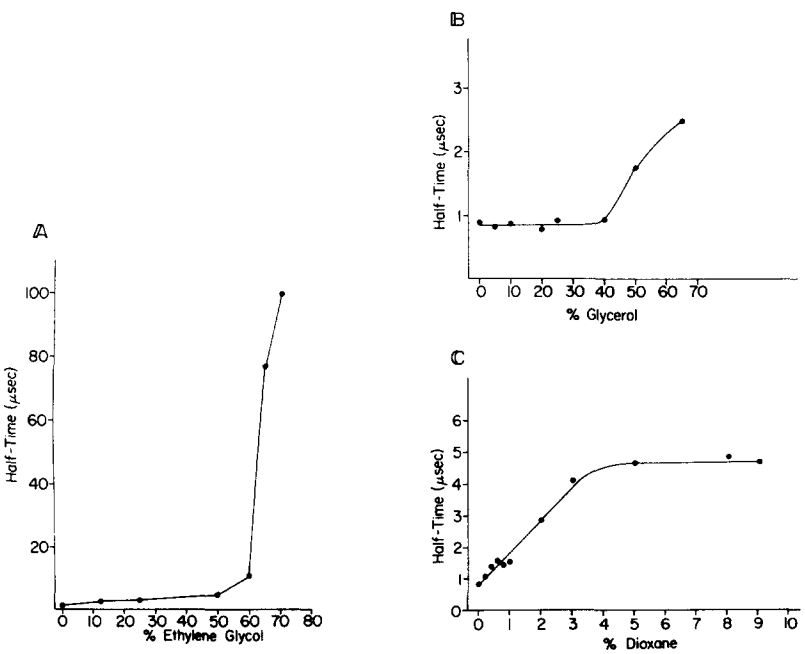

Fig. 7. The effect of substituting water with aqueous mixtures of organic solvents at various concentrations on the half-time of cytochrome *c* oxidation in *R. gelatinosa* at 298 °K.

Mitochondrial electron carriers

Earlier observations by several investigators working with non-photosynthetic systems indicated the participation of water or aqueous phase in biological oxidation-reduction reactions. ^2H_2O and several organic solvents were found to be "non-specific" inhibitors of respiratory chain activity, and the inhibitory action could not be attributed to the solvent viscosity[12]. Whether the reaction was carried out in H_2O or ^2H_2O, no difference was observed in the extent of steady state reduction of the cytochromes $c+c_1$, b, and $a+a_3$ during the transfer of reducing equivalents from substrate to oxygen; only the overall rates were "inhibited" in ^2H_2O at various concentrations[12]. It was observed that in 95% ^2H_2O the rate of succinate oxidation

by O_2 was reduced by 39% and that of NADH oxidation by 46% (ref. 12). Phosphorylation is either not affected or little affected by ^2H_2O (ref. 13). There is an indication that water is required for electrons to flow between cytochromes *b* and c_1, and *a* and a_3 in mitochondria[14]. Also, the existence of a rate-limiting protonolysis in the succinate oxidation system[15], and that of several protonolytic processes at multiple sites in the respiratory chain[12] have been postulated. The lyophilization technique was not employed in these studies. Mainly on the basis of steady state "rate" measurements these workers suggested that the water requirement might be in some way related to a proton movement in electron transfer; however, no mechanisms were formulated.

We have extended our studies further to include a non-photosynthetic material, namely the multi-enzyme system of beef heart mitochondria in which the oxidant for mitochondrial cytochrome *c* is the copper- and iron-containing electron carrier enzyme complex, cytochrome oxidase. The membrane fragment preparation used in the experiments was the light fraction[16] isolated from beef heart mitochondria.

Mitochondrial cytochrome *c* oxidation was observed by means of a combined flow-laser flash system. In this method the respiratory chain components are reduced first by succinate in the presence of CO; the sample becomes anaerobic and the CO complexes of the terminal enzyme cytochrome oxidase are formed. Oxygen-equilibrated water is then rapidly mixed with the sample and 120 ms after the flow stops the laser pulse occurs. In these experiments the 530-nm, 40-ns, 30-mJ output from a frequency-doubled, Neodymium glass Q-switched laser was used. The CO complexes are photodissociated and the reduced terminal enzymes then combine with oxygen more rapidly than with CO[17]. This process results in subsequent oxidation of the various cytochromes in the respiratory chain. The absorption changes were monitored with the post split-beam, dual wavelength readout technique[18]. Oxidation of mitochondrial cytochrome *c* was followed with the 540–550 nm wavelength pair. A control was first run with unlyophilized preparations in 0.2 M manitol–0.05 M–sucrose–0.03 M Tris buffer at pH 7.4 with succinate and glutamate as substrates. The average half-time for oxidation of mitochondrial cytochrome *c* in the control samples was 5.4 ms. The H_2O reconstituted system gave essentially the same results as the control. As in the case of bacterial cytochrome *c*, the substitution of H_2O with ^2H_2O did not affect the extent of the oxidation reaction. The half-time for mitochondrial cytochrome *c* oxidation was obtained, in each case, from about ten separate measurements. The $t_{1/2}$ values were 5.2 ± 1.2 ms in H_2O and 8 ± 1 ms in ^2H_2O. The resulting ratio of the average values $t_{1/2(^2H_2O)}$ to $t_{1/2(H_2O)}$ was 1.5.

The oxidation half-times of cytochrome *b* in the membrane fragments were measured at 560–575 nm with a rapid mixing instrument in a O_2 pulse experiment. The half-time of the reaction in the H_2O reconstituted lyophilized system was about two times slower than the control sample, *i.e.* unlyophilized, suspended in an mannitol–sucrose–Tris buffer (pH 7.6). However, the comparison was made between two sets of reconstituted samples from the same lyophilized preparation which differed only in that ^2H_2O replaced H_2O in one. The flow time in these experiments was 50 ms. Fig. 8 represents the type of traces obtained. These traces are plotted on a semi-logarithmic paper in Fig. 9 to show the isotope effect more clearly. As in the previous cases the extent of cytochrome *b* oxidation was essentially the same in both H_2O and ^2H_2O. The average value for the ratio of half-times obtained in H_2O

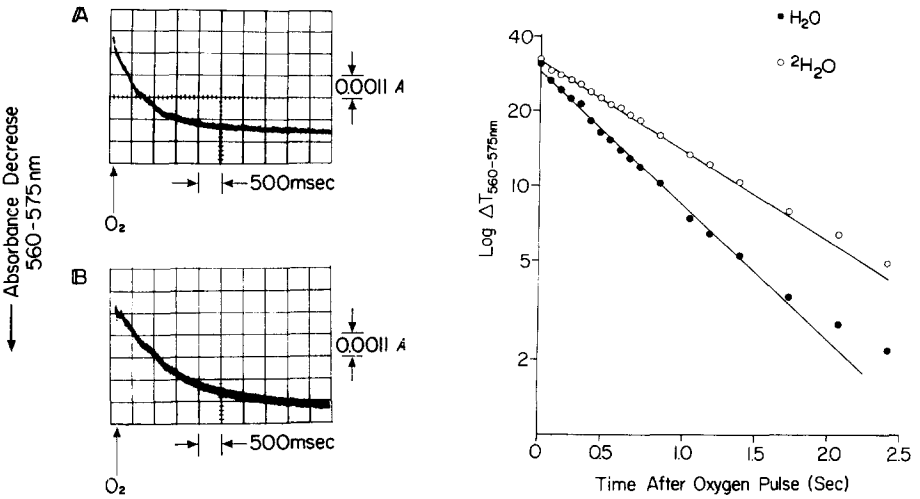

Fig. 8. Typical oscilloscope traces showing the kinetics of mitochondrial cytochrome *b* oxidation (560–575 nm) in membrane fragments prepared from beef heart mitochondria. A suspension of original preparations was lyophilized and stored at −10 °C. 230 mg of the dry mitochondrial powder was reconstituted in 16 ml of distilled water (A) or in 99.81% ^2H_2O (B) prior to the addition of 0.3 ml 1.0 M succinate and 0.2 ml 1.0 M glutamate. 0.04 ml of oxygen-saturated H_2O or ^2H_2O (5.4 μM O_2) was injected from a side syringe into the mixing chamber to initiate oxidation reactions through the respiratory electron transfer chain.

Fig. 9. Semi-logarithmic plot of the traces shown in Fig. 8. The logarithm of reaction amplitude in arbitrary units is plotted on the ordinate against time in seconds after a pulse of O_2 is mixed into the sample.

(0.57 s) and in ^2H_2O (0.85 s) reconstituted systems was found to be 1.49.

The half-times of the reduction reactions of two known electron carriers associated with the mitochondrial fragment preparation were studied in both H_2O and ^2H_2O on a dual wavelength spectrophotometer by the addition of 5 mM succinate to the aerobic sample pretreated with 1.0 mM KCN. The resultant changes were monitored at the wavelength pair 562–575 nm (cytochrome *b*) and 465–500 nm (attributed to a flavoprotein). The average half-time for cytochrome *b* reduction in control samples in an mannitol–sucrose–Tris buffer was 9 s and that for the 465–500 nm change was also 9 s suggesting the possibility that the kinetics of the reactions measured at these wavelengths pairs are governed by a common step within the respiratory chain which is the slowest and thus becomes rate-limiting in both cases. The average half-time for changes monitored at 562–575 nm for H_2O reconstituted samples was 2.7 s and that for the ^2H_2O samples was 3.6 s. The ratio of the average half-time in H_2O to that in ^2H_2O is 1.35.

The kinetics of the changes monitored at 465–500 nm, ascribed to flavoprotein reduction, gave the average half-time value of 2.5 s in H_2O and 3.9 s in ^2H_2O resulting in a half-time ratio of 1.56.

The activation energies for these reduction reactions have been determined at temperatures from 5 to 60 °C and found to be 11.5 ± 1.5 kcal/mole. Preliminary measurements of the activation energy for cytochrome *b* oxidation in the light fraction of beef heart mitochondria indicate that the value is less than 5 kcal/mole.

In summary, the following results from our present work may be emphasized:

(1) Oxidation–reduction reactions of *c*-type bacterial cytochromes do not take place in the absence of water.

(2) Substitution of deuterium for hydrogen in the form of water results in the universal retardation of half-times of various biological electron transfer reactions involving membrane-bound electron carriers in photosynthetic bacteria and mitochondria. The rate-retarding effect is general and is not subject to appreciable variations; the ratio of $t_{½(D_2O)}$ to $t_{½(H_2O)}$ has been found to be close to $\sqrt{2}$ in all cases investigated. Thus, the mass of the hydrogen atom of water may be directly involved in the central mechanism of these reactions.

(3) The $\sqrt{2}$ isotope effect holds throughout the entire temperature range (300 ° to 77 °K) studied for the oxidation of cytochrome *c* in certain photosynthetic bacteria.

(4) The oxidation half-times of bacterial cytochrome *c* are progressively slowed down before the reaction itself is completely inhibited by increasing amounts of ethylene glycol, glycerol and *p*-dioxane in water mixtures.

(5) A low activation energy of 2 to 3 kcal/mole for cytochrome *c* oxidation and a high value, 10–13 kcal/mole, for the reduction of *c*-type bacterial cytochromes and a *b*-type mitochondrial cytochrome have been found.

Possible interpretations

There are several possible explanations of the above experimental results.

The first possibility is that upon replacement of H_2O by ^2H_2O in hydration regions the conformation of the electron carrier molecules is changed in such a way that there is a slight readjustment of the ligands in the vicinity of the heme and that this results in the change in reactivity observed. We find it difficult, however, to understand how this should result in an isotope effect of the same magnitude (*i.e.* $\sqrt{2}$) observed for both oxidation and reduction of the different cytochromes studied. These include four *c*-type cytochromes in four different species of bacteria as well as one *c*-type and one *b*-type mitochondrial cytochrome.

A second possibility is that the intial or final state of the acceptor–donor pair must be prepared before direct electron transfer can take place. This then could involve repolarization of the surrounding environment requiring the movement of protons or, reorientation or readjustment of a hydration region corresponding to a conformational change as the rate-determining step where water rotation is involved. It is difficult, however, to understand on this basis the extremely wide range of absolute values found for oxidation and reduction half-times.

The long-range character of these oxidation–reduction reactions also poses a problem for direct electron transfer theories. An electron tunnelling theory has been proposed to account for the temperature-independent region of the cytochrome oxidation reaction in *Chromatium D* (ref. 19). The results of our experiments, however, suggest that the mass of the hydrogen atom is directly involved in the rate-determining step.

A third possibility is to assume that water is directly involved in the electron transfer process itself and that there exists an array of oriented water molecules between electron carriers as cellular water has been shown to be more structured than pure water[20], and that electron transfer takes place *via* the effective diffusional transfer of a hydrogen atom through a water bridge. The rate-determining step is the basic

diffusional step and involves the average time necessary for water rotation and orbital alignment so that a new water molecule may be formed after the transfer of the hydrogen atom. The results of the substitution experiments involving water soluble solvents may then be understood in terms of the disruptive effect these molecules may have upon the hydrogen-bonded water structure. Quantum mechanical proton tunnelling through hindered rotational barriers by water protons may be important for temperature-independent, *i.e.* zero activation energy, cytochrome oxidation.

There are two physical processes which involve a water structure and water reorientation that may be considered here in order to obtain estimates of the magnitude of the parameters involved in the third possible interpretation of our data. The first is dielectric relaxation in ice[21] and the second is charge conductivity in liquid water and ice[22–24].

In the first case water rotation may be involved and indeed similar results are obtained for the deuterium isotope effect in dielectric relaxation time measurements as we have found for cytochrome reduction. Dielectric relaxation experiments have yielded a temperature-independent deuterium isotope effect of 1.46 (ref. 25) and an activation energy of 13.8 kcal/mole (ref. 26).

Although in the second case of charge conductivity in liquid water a hydrated proton is thought to move by structural diffusion through a water lattice[24], the rate-determining step is presumed to be the liberation and rotation of water molecules until a "hydrogen atom" and lone pair orbital line up to form a hydrogen bond which is part of the new hydration shell of the proton. The hydrogen bonds broken here are "weak" H-bonds of the second hydration shell. It is of interest to note that there is a difference in activation energy observed for water conductivity experiments of 2 kcal/mole in the liquid state[24] and 11–14 kcal/mole (ref. 27) in the solid (ice) state. Possibly the low activation energy found for cytochrome oxidation is a reflection of the breaking of a weak hydrogen bond whereas cytochrome reduction requires the breaking of stronger hydrogen bonds. Experimentally, a value of 1.42 has been found for the ratio of the mobilities of H^+ in H_2O and $^2H^+$ in ^2H_2O in liquid water conductivity experiments[28], and this is taken as support for water rotation as the rate-determining step in water conductivity[23]. A temperature-independent deuterium isotope effect of about $\sqrt{2}$ has also been found for conductivity experiments with HCl–water systems[29].

The activation energies of a large number of diverse inorganic redox reactions involving metal aquo-ions have been found to be near 10 kcal/mole (refs 30–33). This has suggested that these reactions proceed *via* a common mechanism involving water. According to water bridging theories for the mechanism of the iron(II)–iron(III) electron exchange reaction, which does not proceed in the absence of water[31], the electron transfer is effected via a water bridge or water molecules of solvation[30,31,34,35]. A mechanism based on hydrogen atom transfer of this type has been suggested for biological long-range electron transfer reactions[36–38]. The results of the iron(II)–iron(III) electron exchange reaction in ice media studied by Horne[37] indicate that electron transfer can occur over great distances (*e.g.* 100 Å) by a water bridging mechanism.

Of the three possible interpretations of our work presented here we believe that the third, hydrogen atom transfer *via* a water bridge, should be investigated

further as a possible general mechanism for biological oxidation–reduction reactions.

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