The Onset of the Deuterium Isotope Effect in Cytochrome *c* Oxidase†

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ABSTRACT: We have investigated the dynamics of proton equilibration within the proton-transfer pathway of cytochrome *c* oxidase from bovine heart that is used for the transfer of both substrate and pumped protons during reaction of the reduced enzyme with oxygen (D-pathway). The kinetics of the slowest phase in the oxidation of the enzyme (the oxo-ferryl → oxidized transition, F → O), which is associated with proton uptake, were studied by monitoring absorbance changes at 445 nm. The rate constant of this transition, which is 800 s^{-1} in H_2O (at pH ∼7.5), displayed a kinetic deuterium isotope effect of ∼4 (i.e., the rate was ∼200 s^{-1} in 100% D_2O). To investigate the kinetics of the onset of the deuterium isotope effect, fully reduced, solubilized CO-bound cytochrome *c* oxidase in H_2O was mixed rapidly at a ratio of 1:5 with a D_2O buffer saturated with oxygen. After a well-defined time period, CO was flashed off using a short laser flash. The time between mixing and flashing off CO was varied within the range 0.04−10 s. The results show that for the bovine enzyme, the onset of the deuterium isotope effect takes place within two time windows of ≤100 ms and ∼1 s, respectively. The slow onset of the deuterium isotope effect indicates that the rate-limiting step during the F → O transition is internal proton transfer from a site, proposed to be Glu (I-286) (*R. sphaeroides* amino acid residue numbering), to the binuclear center. The spontaneous equilibration of protons/deuterons with this site in the interior of the protein is slow (∼1 s).

In the last step of the respiratory chain, the membrane-bound protein cytochrome *c* oxidase accepts four electrons from reduced cytochrome *c* and transfers them to dioxygen. For every oxygen molecule that is reduced, four protons are taken up and two water molecules are formed. Part of the energy released in this exergonic reaction is utilized to transfer another four protons across the membrane, thus conserving energy in a proton gradient across the membrane (*1*).

The three-dimensional crystal structures of bovine heart (*2, 3*) and *Paracoccus denitrificans* (*4, 5*) cytochrome *c* oxidase have recently been determined. The amino acid sequence of cytochrome *c* oxidase from *Rhodobacter sphaeroides* is highly homologous with that of the bovine enzyme, and the three-dimensional structures of the three core subunits are almost identical in the two enzymes (Svensson-Ek et al., unpublished results).

During turnover, the electrons that are donated by cytochrome *c* are transferred sequentially to the four redox-active cofactors of cytochrome *c* oxidase: Cu_A, heme *a*, and the binuclear center consisting of heme a_3 and Cu_B, at which dioxygen is reduced to water. Since the binuclear center is located in the hydrophobic part of the enzyme, about 35 Å from the proton-input side of the membrane, proton pathways are needed to convey substrate and pumped protons through the enzyme. Putative proton-transfer pathways, consisting of "wires" of protonatable amino acid residues and bound water molecules, have been identified on the basis of studies of site-directed mutants of terminal oxidases (*6, 7*) and the X-ray crystal structures (*2, 4*). One of the pathways, the D-pathway [contains an essential aspartate residue, D(I-132)],[1] leads from solution on the proton-input side to an essential glutamate [E(I-286)] from where the pathway is rather indefinite, but most likely consists of a cluster of water molecules leading toward the binuclear center. Alternatively, after the glutamate, the pathway may lead to the propionates of heme a_3 toward the output side for protons. Therefore, it has been suggested that the glutamate may play an important role in controlling the flow of pumped and substrate (used during reduction of O_2 to water) protons during catalysis. The second pathway, the K-pathway [contains an essential lysine residue, K(I-362)], is shorter and leads directly from the protein surface on the proton-input side to the binuclear center.

The partial steps of the reaction of the solubilized, fully reduced cytochrome *c* oxidase with O_2 can be investigated using the flow-flash technique [for a review, see (*8*)]. The

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[1] Abbreviations: COX, cytochrome *c* oxidase; the kinetic deuterium-isotope effect is defined as the ratio of the rates measured in H_2O and D_2O (k_H/k_D), respectively; R, fully reduced enzyme; A, ferrous-oxy intermediate; P, peroxy intermediate; F, ferryl intermediate; O, fully oxidized enzyme; Amino acid residue and mutant-enzyme nomenclature: E(I-286), denotes glutamate 286 of subunit I.; EQ(I-286), denotes a replacement of glutamate 286 by glutamine; amino acid residues are numbered according to the *R. sphaeroides* sequence.

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reduced enzyme with CO bound to heme a_3 is mixed with an O_2-containing solution. Immediately after mixing, CO is flashed off, which allows oxygen to enter into the binuclear center. With both the bovine and *R. sphaeroides* enzymes, binding of oxygen ($\tau \cong 10$ μs) is followed by oxidation of the two heme groups and formation of the so-called peroxy intermediate (P) at the binuclear center ($\tau = 30-50$ μs) (*9*). Up to this point, there is no uptake of protons. During the following step, one proton is taken up, and the oxo-ferryl intermediate (F) is formed, with a time constant of ~100 μs. This proton uptake triggers the transfer of the fourth electron from Cu_A to heme *a* (*10*). Finally, with a time constant of ~1 ms, this last electron is transferred from heme *a* to the binuclear center concomitantly with the uptake of a second proton and the formation of the oxidized enzyme (O) [(*9*); for a detailed review of the reaction of reduced cytochrome *c* oxidase with oxygen, see (*8, 11*)]. A net of about two protons are taken up during oxidation of the reduced, solubilized enzyme (*9, 12*), and about two protons are taken up upon reduction of the oxidized enzyme (*13*). Both protons that are taken up during oxidation of cytochrome *c* oxidase have been shown to be taken up through the D-pathway [for review, see (*14*)]. The K-pathway, on the other hand, is used for the uptake of at least one of the two protons taken up upon reduction.

The transfer of protons through the D-pathway presumably controls the rate of the last kinetic phase during oxidation of the fully reduced enzyme (F → O transition) (*15*), a reaction step that is coupled to proton pumping (*12, 16*). Earlier studies show that with the bovine enzyme the rate of the F → O transition decreases when water is substituted for deuterium oxide (*15, 17, 18*). In these studies, the enzyme was incubated or turned over in a D_2O-containing buffer prior to investigation of the oxidation kinetics. Consequently, in these experiments protium has presumably been exchanged completely for deuterium, both at the surface groups of the protein and inside the proton-transfer pathways. If, however, the sample is not equilibrated with deuterium oxide prior to mixing of the reduced enzyme with oxygen, the isotope effect may depend on the incubation time in D_2O, which will reflect the proton equilibration dynamics of the proton-transfer (D-) pathway.

In this study, we have investigated the development of the kinetic deuterium isotope effect as a function of the incubation time in D_2O, i.e., the time between mixing the H_2O-equilibrated, fully reduced, CO-bound enzyme solution with an O_2-saturated D_2O solution, and flashing off CO. We found that an increase of the incubation time in D_2O prior to initiation of the reaction with O_2 results in slower F → O transition kinetics. With the bovine enzyme, the appearance of the slower kinetics takes place within two characteristic time frames of ≤100 ms and ~1 s, respectively. The results are interpreted in terms of a model in which the F → O transition is rate-limited by an internal proton transfer from Glu(I-286) to the binuclear center. The equilibration of protons/deuterons with Glu(I-286) is slow (~1 s).

MATERIALS AND METHODS

The bovine cytochrome *c* oxidase was prepared using the method of Brandt et al. (*19*).

Flow-Flash Measurements. Oxidized cytochrome *c* oxidase was diluted in a H_2O solution of 0.1 M Hepes−KOH, pH 7.5, 0.1% dodecyl-β-D-maltoside, and 5 μM PMS (phenazine methosulfate) to a concentration of about 12 μM. Then, the solution was made anaerobic by exchanging the air for nitrogen. The fully reduced enzyme was prepared by an anaerobic addition of sodium ascorbate to a final concentration of 2 mM. Finally, nitrogen was removed, and the enzyme solution was placed under pure carbon monoxide atmosphere.

The solution of the reduced cytochrome *c* oxidase−CO complex was transferred anaerobically to one of the drive syringes (500 μL total volume) of a locally modified stopped-flow apparatus (Applied Photophysics, DX-17MV). The other drive syringe (2.5 mL total volume) contained a D_2O solution of 0.1 M Hepes and 0.05% dodecyl-β-D-maltoside, equilibrated with pure O_2 at 1 atm and adjusted to the same pH-meter reading (pH* = 7.5) as in H_2O.[2]

After mixing, the reaction was initiated by dissociating CO with a ~10 ns, ~100 mJ laser flash (Nd:YAG laser, Spectra Physics). The time between mixing and flashing off CO was controlled with an adjustable delay circuit that was connected between the stop-syringe switch and the laser. The delay times were varied in the range 0.040−10 s. To avoid an acceleration of the CO dissociation rate, care was exercised to keep the actinic light at a low level. Four traces, measured at 445 nm, were averaged for each delay time.

Data Analysis and Calculation of the Isotope Effect. The absorbance changes of the F → O transition in the bovine enzyme at 100% H_2O or ~100% D_2O were recorded at five different wavelengths (432, 445, 580, 605, and 610 nm). These changes were fitted simultaneously using the program Pro-Kineticist (Applied Photophysics Ltd.) as described (*9*). The kinetic deuterium isotope effect was calculated as the ratio of the rates of the first, dominating phase measured in H_2O and D_2O, respectively. The data recorded after various mixing times of the H_2O-equilibrated enzyme with a D_2O solution were evaluated using a program written in Matlab (The Math Works Inc.) as described in detail under Discussion.

RESULTS

As described in the introduction section, after flash-induced CO dissociation from the fully reduced cytochrome *c* oxidase, oxidation of the enzyme takes place in a number of kinetically resolved phases (Figure 1A). At 445 nm, the initial increase in absorbance at $t = 0$ is due to CO dissociation from heme a_3. It is followed by a biphasic decrease due to oxygen binding (formation of intermediate A) and formation of the P intermediate with rate constants of ~10^5 s^{-1} and 3 × 10^4 s^{-1}, respectively. The P → F transition can be seen as a slight increase in absorbance around 0.15 ms after the flash with a rate of ~10^4 s^{-1}, mainly associated with electron transfer from Cu_A to heme *a*, which is controlled by the proton uptake associated with the P → F transition at the

[2] To obtain the pD value, 0.4 should be added to the pH-meter reading with a D_2O solution (*20*). Since the pK_a of protonatable groups shift by about the same value, the protonation level of these groups is about the same in H_2O and D_2O (*20*), respectively, at the same *pH-meter reading*. However, one should remember that with the same pH-meter reading, in D_2O the D^+ concentration is lower than that of H^+ in H_2O. The experiments in this study were done in a pH range in which the pH dependence of the F → O rate is much smaller than the deuterium-isotope effect.

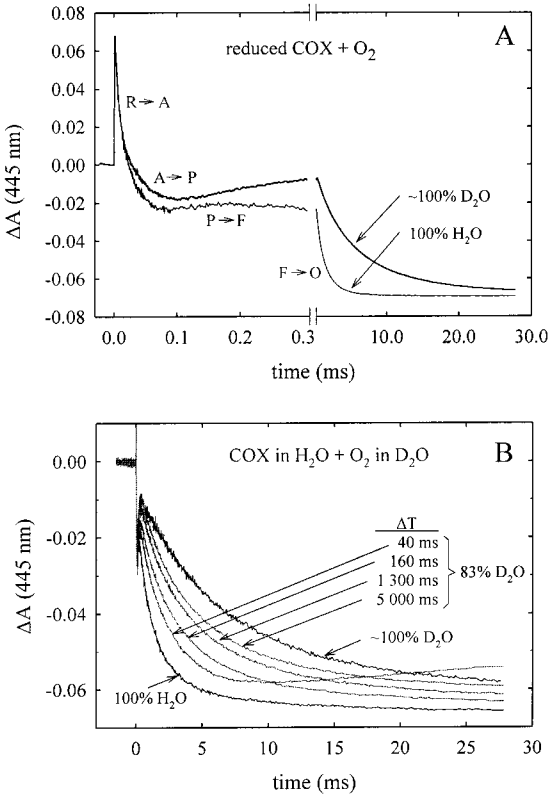

FIGURE 1: Absorbance changes at 445 nm, associated with reaction of the fully reduced bovine cytochrome *c* oxidase with oxygen. (A) Absorbance changes measured with enzyme turned over in H_2O and D_2O, respectively (100% H_2O and ~100% D_2O, respectively, after mixing). The four resolved kinetic phases are indicated (see text). The P → F transition is more pronounced in the D_2O sample, mainly because of the much slower F → O transition that follows the P → F transition. (B) Absorbance changes associated with the F → O transition. An anaerobic sample of reduced CO-bound cytochrome *c* oxidase in 0.1 M Hepes/H_2O buffer, pH 7.5, was mixed rapidly at a ratio of 1:5 with a buffer of the same composition, but made from D_2O. The time (ΔT) between mixing and flashing off CO was varied as indicated in the figure. The absorbance changes obtained when the H_2O-incubated enzyme was mixed with a H_2O buffer and the D_2O-incubated enzyme was mixed with a D_2O buffer, respectively, are also shown. Note that these experiments were done with a different sample as compared to (A). All traces shown are averages of four traces. Experimental conditions [for both (A) and (B)]: 22 °C, 0.1 M Hepes−KOH [100% H_2O or D_2O in (A), 83% D_2O in (B) after mixing], pH-meter reading 7.5, 0.05% dodecyl-β-D-maltoside, 5 μM PMS, 1.3 μM reacting enzyme (the traces have been scaled to 1 μM enzyme), 1 mM O_2.

binuclear center (*10*, *21*). The final decrease in absorbance is associated with the transfer of the fourth electron from the Cu_A−heme *a* equilibrium to the F-intermediate at the binuclear center, forming the oxidized enzyme (O). With the bovine enzyme, absorbance changes associated with the F → O transition were found to display biphasic kinetics with a dominating rate constant of 800 s^{-1} (~80% of the amplitude) and a slower rate of 200 s^{-1} [see also (*9*)].

We studied absorbance changes associated with the F → O transition at five different wavelengths (432, 445, 580, 605, and 610 nm) with enzyme in H_2O and enzyme that was turned over in D_2O prior to the experiment at a pH-meter reading of 7.5 (see the Materials and Methods). In each experiment, the rate constant of the dominating, faster component was determined from a global fit of the data at all five wavelengths. The rate constants for the H_2O and D_2O samples were determined to be $k_H \cong 800$ s^{-1} and $k_D \cong 200$ s^{-1} (at ~100% D_2O with enzyme turned over in D_2O prior to the experiment), respectively (see Figure 1A), and based on measurements with three different samples, the kinetic deuterium isotope effect was found to be 4 ± 1 (SD).

The flow-flash method was also used to investigate the onset of the deuterium isotope effect. One drive syringe of the stopped-flow apparatus contained fully reduced, CO-bound cytochrome *c* oxidase solubilized in an H_2O buffer at pH 7.5, and the other drive syringe contained an oxygenated D_2O buffer at the same pH-meter reading (see Materials and Methods). The two solutions were mixed rapidly at a ratio of 1:5. Consequently, the fraction of D_2O after mixing was 83%. The time (ΔT) between mixing and the CO-dissociating laser flash, that initiates the oxygen reaction, was varied in the range 0.04−10 s, where the upper limit was set by the thermal dissociation of CO from reduced heme a_3 (0.02 s^{-1}) (*22*, *23*).

Figure 1B shows the absorbance data from four experiments in which different delay times between mixing and flash (ΔT) were used. As seen in the figure, the isotope effect increased when the incubation time in D_2O was prolonged. The trace recorded at the shortest delay time ($\Delta T = 40$ ms) had a somewhat different shape as compared to the other traces. This is because for very short delay times the mixing in the flow-flash apparatus was not completed at the time of the laser flash.[3] Therefore, reduced, CO-bound enzyme entered into the cuvette after initiation of the reaction, which resulted in a slight increase of the absorbance at 445 nm.

The same experiments as those described above were also repeated with the *R. sphaeroides* cytochrome *c* oxidase. In contrast to the bovine enzyme, the total maximum isotope effect [at 100% D_2O, the effect was 7 ± 1; see also (*15*)] was reached also for short ΔTs (data not shown), which indicates that in this enzyme the proton-transfer pathway is more accessible to the solution protons (see Discussion).

DISCUSSION

In this work we have investigated the onset of the deuterium isotope effect of the kinetic phase associated with the F → O transition ($k_H \cong 800$ s^{-1} for the bovine enzyme). With 100% D_2O, the kinetic deuterium isotope effect of this phase was found to be 4 ± 1 for the bovine enzyme. This value is higher than that of 2.5, reported earlier (*17*). However, a kinetic deuterium isotope effect of 4.3 was observed upon injection of one electron to cytochrome *c* oxidase in which the F state was preformed by incubation in H_2O_2 (*18*). A higher value of ~7 was observed with the *R. sphaeroides* cytochrome *c* oxidase.

The F → O phase exhibit the largest isotope effect of all the kinetic phases observed during reaction of the fully reduced enzyme with O_2. The deuterium isotope effect is a true kinetic effect (i.e., not due to changes in pK_as) because the reaction was investigated in a pH region (pH 7.5) where the pH dependence of the reaction rate is small.

As seen from the data in Figure 1, the average rate of the F → O transition decreased with increasing incubation time in D_2O. An onset of the deuterium isotope effect on a time

[3] The slow mixing time is due to the relatively low drive-syringe pressure, which had to be used because of the asymmetric mixing (different syringe sizes).

scale ≫1 ms (time constant of the F → O transition) indicates a relatively slow exchange of protons/deuterons with the enzyme and is expected to yield a biphasic behavior with the two components corresponding to enzyme populations equilibrated with protons and deuterons, respectively. A rapid onset of the deuterium isotope effect on a time scale ≪1 ms indicates a rapid exchange of protons/deuterons with the enzyme and is expected to display a monophasic behavior with a rate corresponding to the relative fraction of enzyme equilibrated with protons/deuterons.

Part of the deuterium isotope effect was observed already after the shortest mixing time of 40 ms, but the remaining effect developed on a much slower time scale of ∼1 s (see below). If the effect observed during the first 40 ms develops on a time frame ≫1 ms, the data should be evaluated using a mathematical model based on a sum of two exponential functions (see model I, discussed below). However, we also discuss a model based on a monoexponential behavior (model II) because we cannot exclude that the fractional deuterium isotope effect observed during the first 40 ms is associated with a fractional equilibration of protons/deuterons on a time scale ≪1 ms.

Model I. The equilibration time of H_2O/H^+ or D_2O/D^+ with the sites in the enzyme which are involved in the rate-determining proton-transfer reaction(s) during the F → O transition is much longer than the time constant of the F → O transition (see Figure 3). In other words, we assume that at a given, short time after mixing there are two enzyme populations, one with D_2O/D^+ and one with H_2O/H^+, at the sites involved in proton transfer and the two populations are in a slow (compared to the F → O reaction rate) equilibrium. Therefore, when fitting the traces obtained after different mixing times of the H_2O-incubated enzyme with the D_2O solution, two kinetic components with rates of 800 s^{-1} (enzyme with H_2O/H^+) and 200 s^{-1} (enzyme with D_2O/D^+), respectively, were assumed, and the ratio of the contribution of these components was determined from a fit to the different traces obtained at different delay times, ΔT:

$$f(t)_{\text{model I}} = A_1[(1-\alpha)\exp(-800t) + \alpha\exp(-200t)] + A_3t + A_4 \quad (1)$$

where A_1 is the amplitude of the kinetic phases with rates of 200 and 800 s^{-1}, and α is the relative contribution of the 200 s^{-1} phase. As discussed above, in experiments with pure H_2O or D_2O, the F → O phase is biphasic. Therefore, according to model I, for each of the two populations, two kinetic phases should be used. Since a fit using a total of four phases would not give meaningful numbers, instead of adding additional exponential functions, we only added an additional linear function when fitting the traces (A_3t). This linear component was the simplest function that could be used to account for the contribution from other kinetic phases, and the use of this component was sufficient to obtain satisfactory fits within the noise (<10% residuals, i.e., the difference between the fit and the data). In addition, the linear function accounted for the slow rereduction by excess ascorbate after oxidation of the enzyme

In Figure 2A the normalized fraction of the 200 s^{-1} component (α, i.e., the enzyme population equilibrated with D^+/D_2O) is plotted as a function of incubation time in D_2O.

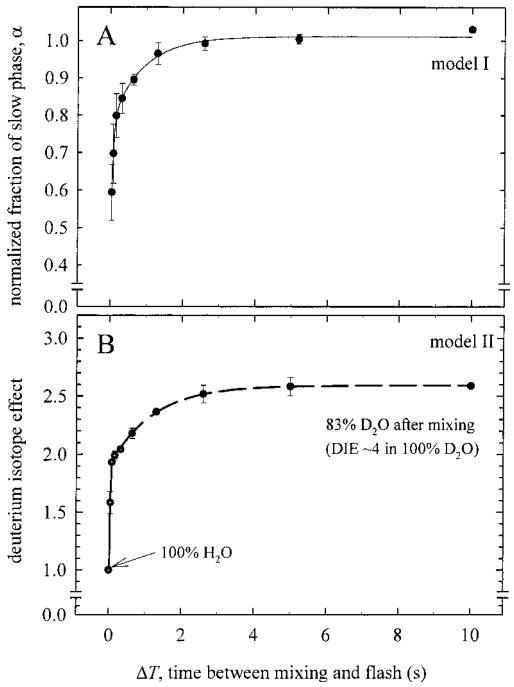

FIGURE 2: (A) Fraction α (see eq 1) of the slow component in the data in Figure 1B (i.e., D^+/D_2O equilibrated interior of the D-pathway) as a function of the incubation time in D_2O, ΔT (model I, see text and Figure 3). Due to the complexity of the fitting procedure (see Discussion), the absolute value of the fraction is not given, and it is normalized to the maximum effect. (B) Kinetic deuterium isotope effect as a function of incubation time in D_2O, ΔT (model II, see text and Figure 3). The kinetic deuterium isotope effects were calculated from ratios of k_1 in eq 2 obtained in 100% H_2O and k_1 measured after the different incubation times (data from Figure 1B). The experimental conditions were as described in the legend of Figure 1. The error bars are standard errors from two independent runs (different samples) with four traces in each run. The solid lines are fits of the data with models I [in (A)] and II [in (B)], respectively. The dashed line indicates a ΔT region where model II does not apply.

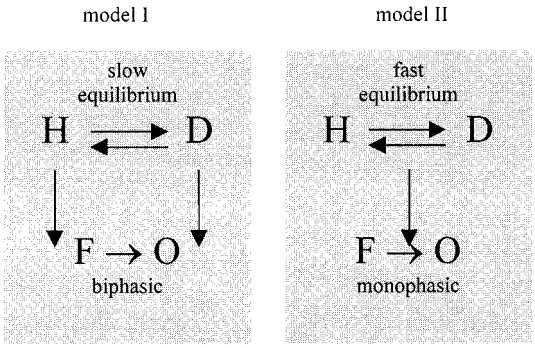

FIGURE 3: Schematic representation of the two different models used for evaluation of the experimental data. According to model I, the equilibration time of the group(s) [presumably E(I-286)] that donate(s) a proton to the binuclear center during the F → O transition is much slower than the time constant of the F → O transition itself. In other words, at a given, short time after mixing of the H_2O-equilibrated enzyme with the D_2O solution, there are two enzyme populations, one in which the group is protonated and one in which it is deuterated. According to model II, the equilibration time of the group is faster than the time constant of the F → O transition. Biphasic and monophasic F → O kinetics are expected for models I and II, respectively.

First, there is a rapid increase in α, and with an incubation time of 100 ms, α is ∼0.5. It is followed by a slower increase with a time constant of about 1 s.

Model II. The equilibration rate of H_2O/H^+ or D_2O/D^+ with the sites involved in the rate-limiting step of proton transfer is faster than the F → O transition (see Figure 3). This scenario is only valid for the shortest times between mixing and flash. The assumption implies only one kinetic component with a rate that decreases with increasing incubation time in D_2O. When fitting the data, we used the function shown in eq 2:

$$f(t)_{\text{model II}} = A_1[0.8\exp(-k_1t) + 0.2\exp(-k_2t)] + A_3t + A_4 \quad (2)$$

The amplitude fraction corresponding to the faster of the two F → O components (with a rate constant k_1) accounted for about 80% of the total F → O absorbance change (amplitude A_1), and this value was fixed for all mixing-to-flash times (ΔT). The linear function was added to account for the slow rereduction by excess ascorbate after oxidation of the enzyme. In Figure 2B, k_1 in eq 2 is plotted as a function of the delay time ΔT. A similar graph to that seen in Figure 2A was obtained; the kinetic deuterium isotope effect increased to about 2 in a rapid phase with a time constant ≤100 ms, followed by a slower increase to a kinetic deuterium isotope effect of 2.6 with a time constant of about 1 s. The maximum value of 2.6 at 83% D_2O is consistent with the kinetic deuterium isotope effect of ∼4 at 100% D_2O when applying the theory of the "proton-inventory technique" (*24*−*26*). According to this theory, the ratio of the rates of a proton-transfer reaction measured at a fraction, χ, of D_2O in solution, $k(\chi)$, and in pure H_2O, $k(0)$, for a case where the entire kinetic deuterium isotope effect is associated with a single rate-limiting proton-transfer step (which is the case for the F → O transition; Karpefors, Ädelroth, and Brzezinski, *Biochemistry*, in press) is:

$$\frac{k(\chi)}{k(0)} = 1 - \chi + \chi\sigma \quad (3)$$

where $\sigma = k(1)/k(0)$, i.e., the inverse of the kinetic deuterium isotope effect at 100% D_2O, and we have assumed that under equilibrium conditions the group involved in the rate-limiting proton-transfer step displays the same isotopic enrichment as a water molecule in the bulk solution (*24*).

Discrimination between Models I and II and the Origin of the Biphasic Onset of the Deuterium Isotope Effect. The fact that we observe deuteration of a protonatable site(s) in the enzyme with a time constant of about 1 s implies that model I should be used. However, the presence of a rapid (≤100 ms) component indicates that deuteration can occur to some extent also on a shorter time scale. If this time scale is shorter than 1 ms (time constant of the F → O transition), model II should be used. Below, we discuss possible origins of the biphasic equilibration kinetics in light of earlier results from studies of mutant forms of cytochrome *c* oxidase from *R. sphaeroides* and discuss the application of the two models.

In the flow-flash experiment, during the P → F and F → O transitions, protons are transferred exclusively through the D-pathway [(*21*, *27*); for review, see (*14*)]. During the P → F ($\tau \cong 100\ \mu$s) transition, a proton is first transferred to the binuclear center from a group in the vicinity, proposed to be E(I-286) (*15*, *28*), followed by rapid reprotonation of E(I-286) from the bulk solution, concomitantly with internal electron transfer from Cu_A to heme *a* (*28*). Consequently, the rate-limiting step for the P → F transition is the internal proton transfer to the binuclear center. Recent results from studies of the ED(I-286) mutant enzyme showed that this mutant enzyme displays a kinetic deuterium isotope effect for the F → O transition of 2.5 as compared to ∼7 for the wild-type enzyme (Ädelroth, Karpefors, Gennis, and Brzezinski, unpublished data), which indicates that E(I-286) is involved in proton transfer also during the F → O transition. Experimental studies using Fourier transform infrared spectroscopy indicate that E(I-286) is in the protonated state at pH 7.5 (*29*−*31*). Even though the rates of the P → F and F → O transitions differ by a factor of ∼10, proton transfer from the same group [E(I-286)] may be rate-limiting for both transitions because the driving force for the internal proton transfer may be different in the two reaction steps [see, e.g., (*8*)].

On the basis of the experiments discussed above, we discuss two scenarios that explain the biphasic onset of the deuterium isotope effect.

Case 1: We assume that deuteration of E(I-286) is responsible for the observed decrease in the F → O rate with increased delay time after mixing. The fact that we observe a deuteration component with a time constant of ∼1 s implies that the deuteration of E(I-286) is slow (cf. model I). To explain the presence of a faster deuteration component (see Figure 2A), we note that the exchange of protons in the D-pathway before the F → O transition not only is due to passive diffusion into the pathway, but also is due to an active uptake of substrate and presumably also pumped protons during the P → F transition ($\tau \cong 100\ \mu$s), that precedes the F → O step. If E(I-286) is deuterated in a fraction of the enzyme population during the P → F phase, in the following F → O phase, already after the shortest delay time, a fraction of the enzyme will have a deuterated E(I-286) and the remaining fraction will have protonated E(I-286) [only a fraction of E(I-286) is deuterated because there are several water molecules in the D-pathway (see, e.g., (*32*, *33*), some of which may have slowly exchangeable protons with the bulk solution]. In other words, according to this scenario, the deuteration of E(I-286) is inherently slow ($\tau \cong 1$ s), and the rapid component in the appearance of the deuterium isotope effect is due to an active proton uptake during the P → F transition.

Case 2: Alternatively, the biphasic development of the deuterium isotope effect may be a consequence of rapid deuteration of surface groups, before equilibration of protons with the protein interior. Earlier results with the *R. sphaeroides* enzyme show that mutation of D(I-132), near the entry point of the D-pathway, to its nonprotonatable analogue results in dramatically decreased enzyme activity (*7*) and the reaction stops after formation of the F intermediate (*28*). This shows that in the DN(I-132) mutant enzyme, proton transfer to the mouth of the D-pathway can become rate-limiting for the F → O transition if the delivery of protons from the bulk is slow enough. It is therefore possible that in the experiments discussed in the present work, within a time window in which E(I-286) is still protonated (not deuterated) and residues around D(I-132) are deuterated, the proton uptake into the D-pathway is the rate-limiting step for the F → O transition. After ∼1 s, when E(I-286) becomes deuterated, again the deuteron transfer from E(I-286) to the binuclear center is

the rate-limiting step for the F → O transition. Since the equilibration of sites close to the protein surface is likely to be rapid, after the shortest time between mixing and initiation of the reaction, monophasic kinetics are expected for the F → O transition (a rate of 410 s^{-1} was found if model II was applied). Consequently, according to this scenario, biphasic kinetics should be assumed (i.e., model I still applies) for the F → O transition, but the faster of the two components should be fixed at a rate of 410 s^{-1}, i.e., the rate of proton uptake to the D-pathway with deuterated entry sites.

In summary, both scenarios discussed above (cases 1 and 2) explain the presence of a rapid phase in the appearance of the deuterium isotope effect and imply that model I best models the experimental data. However, according to case 1, the rapid component should be fixed at 800 s^{-1} [see eq 1, a fraction protonated (H^+) E(I-286) in slow equilibrium with the bulk solution] whereas according to case 2 the rapid component should be fixed at 410 s^{-1} (instead of 800 s^{-1} in eq 1, rapid deuteron/proton exchange of the protonatable group with the bulk solution).

Both cases gave about the same results (in Figure 2A, the solid line is a fit of the data with a mathematical model based on case 1).

Deuteration Kinetics in the R. sphaeroides Enzyme. In *R. sphaeroides*, the full deuterium isotope effect for the F → O transition was observed also after short delay times between mixing and flash-induced dissociation of CO. This observation is not inconsistent with the same proton-transfer mechanism being utilized in the *R. sphaeroides* as in the bovine enzyme. In the bovine enzyme, the *spontaneous* proton-exchange time constant of E(I-286) with the bulk solution of ~1 s is incidental and has nothing to do with the proton-uptake mechanism *during turnover*, which is rapid (~1 ms). The fact that this spontaneous proton exchange is relatively slow in the bovine enzyme makes it possible to use the flow-flash technique to investigate the proton-transfer mechanism, as was done in the present study. In the *R. sphaeroides* enzyme, the *spontaneous* proton exchange is incidentally faster, which reflects a faster proton exchange between the bulk phase and the D-pathway. As indicated above, this does not exclude the same proton-uptake mechanism *during turnover* in the *R. sphaeroides* and bovine enzymes.

Summary. In summary, we have shown that in the bovine enzyme the onset of the deuterium isotope effect during the F → O transition is relatively slow (~1 s), which indicates that the proton used during the F → O transition at the binuclear center is taken from an internal group in the D-pathway. On the basis of results from other studies, this group is proposed to be E(I-286) (*15*, *21*).

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