Intramolecular Electron Transfer in Cytochrome *o* of *Escherichia coli*: Events following the Photolysis of Fully and Partially Reduced CO-Bound Forms of the bo_3 and oo_3 Enzymes†

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ABSTRACT: The events which follow photolysis of CO-inhibited fully reduced and CO-bound mixed-valence cytochrome *o* have been studied in two variants of the enzyme, one of which contains heme B at the low-spin site (bo_3) and the other of which contains heme O (oo_3). For this, isolated enzyme was prepared from three different strains of *Escherichia coli* which produce these two variants in different relative amounts [Puustinen, A., Morgan, J. E., Verkhovsky, M., Thomas, J. W., Gennis, R. B., & Wikström, M. (1992) *Biochemistry 31*, 10363–10369]. In both types of enzyme microsecond electron redistribution was observed from the oxygen-binding heme to the low-spin heme. In the bo_3 enzyme, the rate was similar to that in the bovine enzyme (3 μs), but in the oo_3 enzyme, it was several times slower. However, in both types of cytochrome *o*, the same electron redistribution process was also apparently observed on other time scales, some faster and some slower. The rate of CO rebinding in the mixed-valence enzyme was found to be slower than in the fully reduced enzyme, apparently because of the subpopulation of oxidized oxygen-binding heme produced by the electron redistribution. The extent of this electron redistribution, and thus the inter-heme ΔE_m, can be calculated from this change in rate. The heme B and heme O containing low-spin sites have E_m values about 20 and 50 mV lower, respectively, than the oxygen-binding heme.

Cytochrome *o*, one of the two terminal respiratory oxidases of *Escherichia coli*, has recently been shown to be closely homologous to the family of cytochrome *c* oxidases and, like them, to be a proton pump [see Saraste (1990)]. These findings, combined with the availability of site-directed mutants, have made cytochrome *o* a major focus for the study of terminal oxidases.

Cytochrome *o* also bears a strong resemblance to cytochrome *c* oxidase in terms of its redox centers: It contains a bimetallic (heme–copper) site for oxygen reduction and an additional low-spin heme, which serves as the immediate electron donor to this catalytic site. In (most) cytochrome *c* oxidases, both of the hemes are heme A, whereas in cytochrome *o*, the oxygen-binding heme is heme O, and the low-spin heme may be heme O or heme B (Puustinen et al., 1992). The only departure from the cytochrome *c* oxidase redox carrier motif is that the Cu_A center is not present in cytochrome *o* (it is also not found in the related quinol oxidase, cytochrome aa_3-600 from *Bacillus subtilis*). This is consistent with the fact that these enzymes take their electrons, not from cytochrome *c*, but from quinol, a two-electron donor [see also Lauraeus et al. (1991)].

The two aspects of terminal oxidases which have most intrigued enzymologists are (1) the mechanism which catalyzes the reduction of oxygen to water and (2) the way energy from this process is saved as an electrochemical proton gradient, and in particular the role played by proton pumping. It is clear that a good understanding of electron transfer reactions in these enzymes is a prerequisite to elucidating these processes.

The most direct method which has been found to study intramolecular electron transfer in cytochome *c* oxidase is to observe electron redistribution after flash photolysis of partially reduced CO-bound forms of the enzyme. This method takes advantage of the fact that a well-characterized CO-bound mixed-valence (COMV)[1] compound of the enzyme can be prepared in which the heme and copper of the oxygen-reduction site are reduced while the remaining metal centers are oxidized. In bovine cytochrome *c* oxidase, photolysis of Fe–CO in this COMV compound results in fast (τ = 3 μs) redistribution of electrons from the oxygen-binding heme (Fe_{a3}) to the low-spin heme (Fe_a), followed by slower (τ = 35 μs) redistribution of electrons from these two hemes to Cu_A (Boelens et al., 1982; Morgan et al., 1989; Oliveberg & Malmström, 1991; Verkhovsky et al., 1992).

Since the Cu_A center is not present in cytochrome *o*, the second of these two processes cannot occur in this enzyme. But, it seems likely that a process corresponding to the fast inter-heme electron transfer does take place, since the hemes of this enzyme are very similar in function and protein environment to those of cytochrome *c* oxidase. In fact, evidence for such inter-heme electron redistribution in cytochrome *o* has been presented (Brown et al., 1993).

In this paper we report a detailed study of the events which follow photolysis in cytochrome *o*. An inter-heme electron transfer process, similar to what is found in cytochrome *c* oxidase, has been characterized. This process has been studied in variants of the enzyme which contain widely different

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[1] Abbreviations: bo_3, cytochrome *o* in which the low-spin heme is heme B and the oxygen-binding heme is heme O; COFR, CO-bound fully reduced enzyme; COMV, CO-bound mixed valence enzyme; E_m, redox midpoint potential (v. Normal Hydrogen Electrode); MES, 2[N-morpholino]ethanesulfonic acid; LM, lauryl maltoside; oo_3, cytochrome *o* in which both the low-spin heme and the oxygen binding heme are heme O; τ, first order relaxation time constant ($t_{1/e}$).

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relative amounts of heme B and heme O at the low-spin site, in order to assess the effect of this variability on the thermodynamics and kinetics of the process.

MATERIALS AND METHODS

Bacterial Strains and Enzyme Preparation. Cytochrome *o* was purified from three different *E. coli* strains. GO103 (cyd⁻; Oden et al., 1990) is comparable to wild type in that the genes encoding cytochrome *o* are intact in the chromosome; RG145 (cyd⁻; Au & Gennis, 1987) produces 5–10 times more cytochrome *o* than GO103; E286Q is a (subunit I) mutant (Thomas et al., 1993). Bacterial growth and isolation and purification of the enzyme, as well as properties of the enzyme from different strains, have been described previously (Puustinen et. al., 1992).

Kinetic Measurements. Microsecond transient absorption measurements were carried out using an apparatus built around the chassis and monochromator of an Aminco DW-2 spectrophotometer. The normal light path of the Aminco was used, with the following changes: The tungsten–halogen lamp was retained, but the deuterium lamp was replaced with the bulb and reflector of a camera type xenon flash lamp. The light chopper has been halted in a position where the light from only one monochromator passes through the sample (the other beam was blocked). The original sample holder was used in the position furthest from the photomultiplier tube. A frequency-doubled YAG laser was used for photolysis, and the laser beam was brought into the sample compartment through one of the two round ports. To minimize scattering of laser light, the path of the laser to the cell holder was enclosed by a black paper tube; beyond the sample, this tube tapers to a point. A series of slits also shields the detector from scattered light, and optical filters were used to prevent entry of laser light into the photodetector. As in the original instrument, light leaving the sample cuvette was not refocused; the collimated beam simply falls on the photocathode a few centimeters away.

Other details of our instrumental setup were as follows. Xenon flash lamp: Commercial camera-top flash units were used (Braun 370BVC, Braun 380BVC, Braun 400M logic, Olympus) with an external capacitor charger (a modified Model 880, Novatron, Dallas, TX). Optical isolators were used to connect the open collector flash trigger circuits to the TTL timing circuits. Optical filters: For measurements in the blue (Soret) region, a BG3 filter was used. For measurements in the yellow/red (α-band) region, cutoff filters were used, often together with other filters to block sample fluorescence (a cell containing copper sulfate solution was frequently used for this purpose.) Photomultipliers: 9659QB and 9202A selected for enhanced red response (Thorne EMI, U.K.). Special high current capacity voltage dividers with 22 kΩ/stage (2*R* on the first stage and diodes instead of resistors in the final two stages) were used to minimize photomultiplier saturation artifacts. Timing: CTM–05 counter–timer board (Metrabyte). Data acquisition: PCIP-SCOPE 20-MHz oscilloscope board (Metrabyte) and DA90 acquisition software (Alexander Drachev, Tempe, AZ). Data analysis software: Graphic Interactive Management (GIM, Alexander Drachev).

Anaerobic samples were made using standard gas-exchange techniques (Morgan, 1989). Argon (99.99% purity) and CO (99% purity) were passed over manganese oxalate/vermiculite oxygen-scrubbing columns before use. Samples of the COMV enzyme were made by allowing the enzyme to be reduced by CO over the course of several hours (Bickar et al., 1984). To avoid redox cycling and formation of oxygen intermediates,

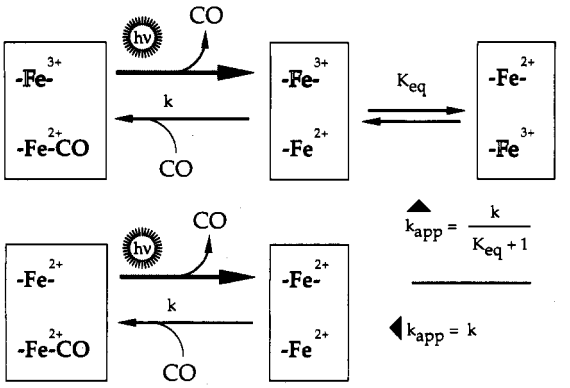

FIGURE 1: Photolysis and rebinding of CO from cytochrome *o*: a comparison of mixed-valence (top) and fully reduced (bottom) forms of the enzyme. Each box represents one state of the enzyme, where the upper Fe is the low-spin heme and the lower Fe is the oxygen-binding (and CO-binding) heme. K_{eq} is the equilibrium constant for electron distribution between the two hemes, while k is the pseudo-first-order rate constant for CO binding to forms of the enzyme where the oxygen-binding heme is in its reduced state.

samples were first made anaerobic with argon before CO was introduced. Since the formation of the COMV compound is slow under low-pH conditions, in some cases it was first made in concentrated samples at higher pH (typically pH 8.0) and then diluted by anaerobic syringe transfer into lower pH buffer which had already been made anaerobic and saturated with CO (Morgan, 1989). Reduced samples were made using dithionite, although the enzyme solutions were made anaerobic before addition of the reductant to minimize side reactions.

The spectra in Figure 4 were constructed from experimental data using classical methods by which the redox state of the oxygen-binding heme is clamped by means of an externally added ligand (Vanneste, 1966). Thus, for example, the reduced minus oxidized spectrum of the low-spin heme can be constructed by subtracting the spectrum of the COMV enzyme from the spectrum of the COFR enzyme. The spectrum of the oxygen-binding heme can then be constructed by subtracting the reduced minus oxidized spectrum of the low-spin heme (above) from the reduced minus oxidized spectrum of the unliganded enzyme. This method is explained in detail in Blair et al. (1982).

RESULTS

The high-spin heme site of a terminal oxidase, in its reduced form, can bind CO in place of oxygen. CO raises the redox potential of the heme, making it possible to form a well-defined CO-bound mixed-valence (COMV) compound of the enzyme in which the oxygen binding heme and Cu_B are in the reduced form while the low-spin heme remains oxidized (Boelens et al., 1982). The iron–CO bond is reversibly photolabile: CO can be repeatedly photolyzed, after which it will rebind to the reduced heme on a time scale of milliseconds (at 1 atm of CO). It has been reported, however, that after photolysis of the COMV compound in cytochrome *c* oxidase there is rapid electron redistribution from the oxygen binding heme to the low-spin heme center (see the introduction). As illustrated in Figure 1, this will decrease the population of reduced oxygen-binding heme and should thus decrease the rate of CO rebinding. Moreover, the extent to which this rate decreases should depend on the heme–heme redox equilibrium constant.

It has recently been shown (Puustinen et al., 1992) that cytochrome *o* produced by different strains and under different growth conditions shows variations in the heme composition at the low-spin site ranging from almost completely heme B (GO103, pseudo-wild type strain) to about 70% heme O

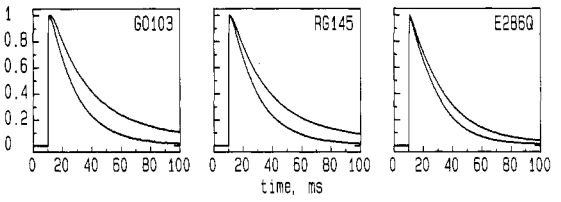

FIGURE 2: The influence of low-spin heme composition on CO recombination kinetics in CO-bound mixed-valence cytochrome *o*. Kinetic traces at 430 nm for CO photolysis and recombination in COFR and COMV enzymes are shown for cytochrome *o* from three different strains of *E. coli* which have widely different ratios of heme B to heme O. Each box shows the data for one strain (labeled in the corner); the upper trace is for COMV, while the lower one is for COFR. The initial jump, at about 10 ms on the time scale, is the CO photolysis, initiated by a laser flash. In all cases, the amplitude of this initial jump has been normalized to 1: cytochrome *o* from GO103, 2.2 μM; RG145, 4.2 μM; E286Q, 7.4 μM; 22 °C; CO, 1 atm; medium, 50 mM MES (pH 6.5) and 0.01% LM .

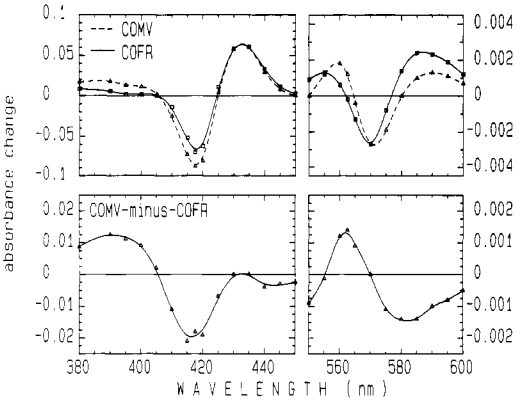

FIGURE 3: Kinetic spectra of the CO-recombination phase showing differences between CO-bound mixed- valence and fully reduced cytochrome *o* from the GO103 strain. The top two panels show spectra for the COMV (- -△- -) and COFR (–□–) enzymes. The bottom two panels show the difference between the COMV and COFR spectra. The amplitudes were obtained from a single exponential fit to the kinetic data. The spectra are divided into left- and right-hand boxes corresponding to Soret band and α-band heme absorptions. A larger magnification is used on the right side because α-band extinction coefficients are smaller. Lines through the points were made by using a spline function. The discontinuity between the two boxes is a region in which we cannot measure because it is too close to the excitation laser wavelength (532 nm): cytochrome *o*, 2.2 μM (for other conditions, see Figure 2).

(E286Q strain). The rate of CO binding to the reduced forms of these different types of enzyme should be similar, since the oxygen-binding heme is always heme O. However, as described in Figure 1, the kinetics of CO rebinding to the mixed-valence enzyme might reveal differences in the redox potentials of hemes B and O at the low-spin site, since we would expect more electron transfer to the centers with higher redox potential, which should be reflected in slower CO rebinding.

Figure 2 shows the time course of CO recombination in reduced and mixed-valence forms of the enzyme for three types of cytochrome *o* which have different ratios of heme B to heme O at the low-spin site. The rate of CO binding in the reduced form is indeed constant, but CO recombination is significantly slower in the COMV compound of all three forms of enzyme. The largest difference is seen in the GO103 enzyme, which contains predominantly heme B at the low-spin site, while the smallest difference is seen in the E286Q enzyme, which contains predominantly heme O. In the RG145 enzyme, the heme composition of which (at the low-spin site) is roughly half-and-half hemes O and B, the time course of CO rebinding is intermediate between those for the other strains.

We have not attempted a quantitative analysis of CO rebinding to mixed valence enzyme in samples which contain both bo_3 and oo_3 enzymes. Samples of this kind would be expected to exhibit biphasic kinetics, but as Figure 2 shows, even large changes in heme composition result in relatively small changes in the time course of CO rebinding. This indicates that the recombination rates for the two types of enzyme are too close to be resolved with any accuracy from such a biphasic curve.

We decided that it would be fruitful to look at the spectra of these processes in the hope of finding more evidence for the putative electron redistribution. In the reduced enzyme, rebinding of CO to the heme should be the only process which contributes to the kinetic phase we observe, but as the scheme in Figure 1 shows, in the mixed valence enzyme, this phase should also include the return of any electrons which left the oxygen-binding heme after CO photolysis. Thus, if the slower recombination rates in the mixed valence enzyme (above) are in fact due to redistribution of electrons, we would expect the spectrum of the CO-rebinding phase for the COMV enzyme to be different from that for the reduced. The difference between these two spectra should reflect this electron redistribution.

Figure 3 shows kinetic spectra of the CO recombination process for fully reduced and mixed valence forms of the enzyme from the GO103 strain, together with the difference spectrum (lower panels). In order to assign this difference spectrum (the putative electron transfer), we also constructed spectra corresponding to all possible one-electron redox events in the enzyme (Figure 4). These include reduced minus oxidized spectra of the oxygen-binding heme and the low-spin heme and double-difference spectra corresponding to electron transfer from one heme to the other [i.e., reduction of one heme with concomitant oxidation of the other; see Verkhovsky et al. (1992)]. The upper group contains spectra for the GO103 enzyme, in which the low-spin site contains predominantly heme B, while the lower group contains spectra for the E286Q enzyme where heme O predominates at the low-spin site. As we have shown previously (Puustinen et al., 1992), the spectra of the o_3 heme from the two strains are very similar, while the spectra of the low-spin hemes O and B are quite different. As a result, the inter-heme electron-transfer spectra for the two kinds of enzyme are significantly different in both the α-region and the Soret region.

The difference spectrum in Figure 3 is clearly similar to the double-difference spectrum for the GO103 enzyme (Figure 4, top), indicating that there is electron redistribution after photolysis of the mixed-valence enzyme and that this redistribution is primarily electron transfer from the oxygen-binding heme to the low-spin heme, a process which has been reported in cytochrome *c* oxidase (Oliveberg & Malmström, 1991; Verkhovsky et al., 1992).

This demonstrates that an electron redistribution occurs in the mixed-valence enzyme, but in order to observe the electron-transfer process itself, it is necessary to study the events which follow photolysis on a faster time scale. Figure 5 shows absorbance changes after CO photolysis covering a time range of microseconds to tens of milliseconds. The traces have been drawn on a multiple-sweep time axis so that all phases present can be seen, even if they have very different time constants. Kinetic traces for two different wavelengths are presented: one to highlight CO photolysis and rebinding and another to highlight electron redistribution processes.

The spectra in Figure 3 show that at 430 nm the contribution of CO photolysis and rebinding is large, while that of an inter-heme electron transfer is small. Consistent with this, the

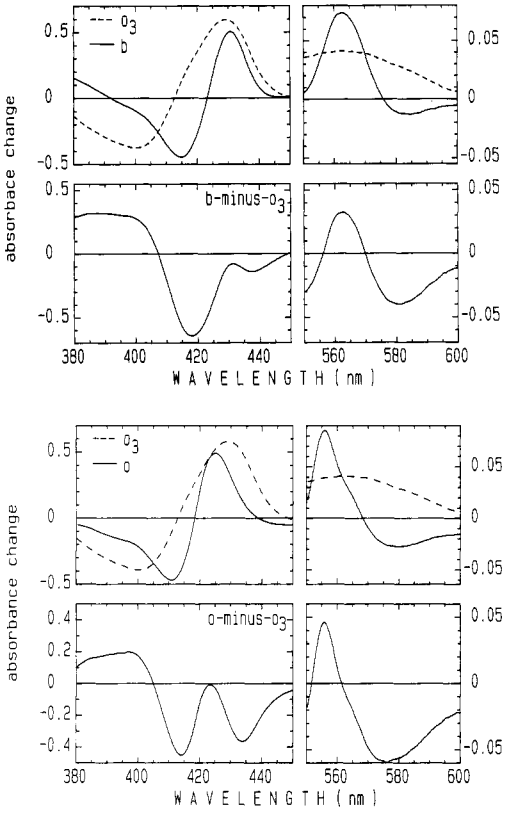

FIGURE 4: Spectra constructed to illustrate all possible one-electron redox events in cytochromes bo_3 and oo_3. (The format has been chosen to allow easy comparison with the kinetic spectra in the other figures.) The top half (top four panels) contains spectra for the enzyme from the GO103 strain, the composition of which is primarily bo_3, while the bottom half (bottom four panels) contains spectra for the enzyme from E286Q, which is at least 70% cytochrome oo_3. Within each half of the figure, the upper two panels show reduced minus oxidized spectra of the two hemes individually, while the lower two panels show a double-difference spectrum corresponding to electron movement from the oxygen-binding heme to the low-spin heme. Spectra were constructed from experimental data using classical "ligand" methods (see Materials and Methods): cytochrome *o* of both strains, 5.5 μM (for other conditions, see Figure 2).

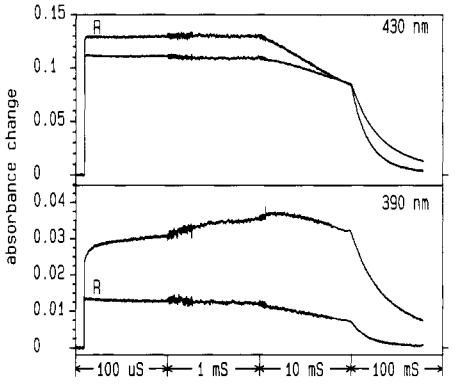

FIGURE 5: Absorbance changes from CO photolysis to rebinding on a four-sweep time scale. The upper panel shows traces for 430 nm, for COFR (marked "R") and COMV, and the lower panel shows traces for 390 nm. All enzymes were from the GO103 strain. The initial jump corresponds to CO photolysis initiated by a laser flash. The data were acquired on five different time scales. Small arbitrary adjustments have been made to reconcile the amplitudes of the partial sweep data; the amplitudes of the fastest sweeps were used for reference: cytochrome *o* of from GO103, 5.4 μM (for other conditions, see Figure 2).

kinetic traces at 430 nm (Figure 5, top) are dominated by two processes: a fast increase in absorbance, which we do not resolve, corresponding to the photolysis of CO, followed by a return to the original absorbance level as CO rebinds. (Note

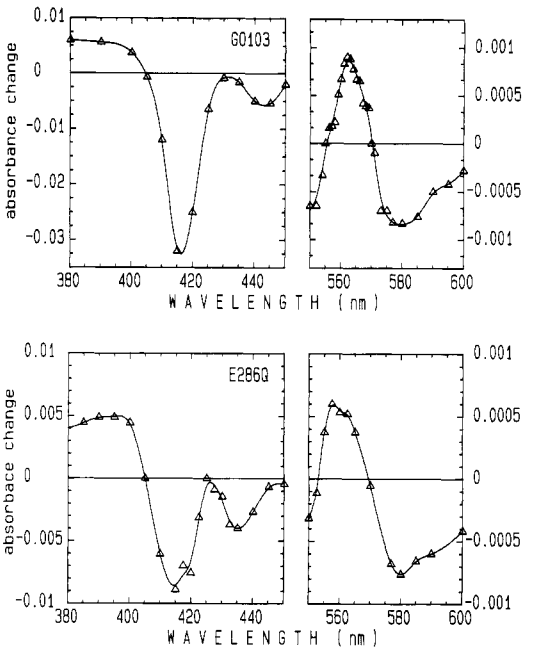

FIGURE 6: Kinetic spectra of the microsecond phase for COMV cytochrome *o* from GO103 (upper two panels) and E286Q (lower two panels) strains. Amplitudes reflect the faster component of a two-exponential fit to the first 100 μs of the reaction: cytochrome *o* of both strains, 4.2 μM (for other conditions, see Figure 2).

that the points of sharp change in the curves arise from the multiple time scale presentation and not multiphasicity in the processes.) The only major difference between the traces for the reduced and mixed-valence samples is the time constant for CO rebinding, as described above. The photophysical events involved in CO photolysis take place on a time scale of picoseconds (Einarsdóttir et al., 1992), far faster than our instrument is able to resolve.

At 390 nm, in contrast, the contribution of CO binding is smaller, and that of intra-heme electron transfer is relatively larger. At this wavelength, the reduced and mixed-valence traces (Figure 5, bottom) are markedly different. There is a clearly resolved phase of absorbance increase in the mixed valence trace (τ = 3 μs), which is not seen in the reduced enzyme. This is followed by at least two more phases of absorbance increase, one on a scale of hundreds of microsecond and one on a scale of milliseconds. These are followed by the CO-rebinding phase. There appear to be some absorbance decreases in the fully reduced sample which are not part of either the photolysis or the rebinding phase. In addition to this there is also a significant difference in the amplitude of the unresolved absorbance jump which accompanies photolysis.

Here again, we constructed spectra in order to study the nature of the different phases. Figure 6 shows spectra of the first resolvable phase for COMV enzyme from the GO103 and E286Q strains. The measured rate constants were close to 3 μs for the GO103 enzyme but somewhat slower (8 μs < τ < 12 μs) for the E286Q enzyme. The GO103 kinetic spectrum is very similar to the "*b* minus o_3" reference spectrum in Figure 4, indicating that this phase represents electron redistribution from the oxygen-binding heme o_3 to the low-spin heme B of this enzyme. However, the kinetic spectrum for E286Q appears to contain approximately equal contributions from both the "*o* minus o_3" and the "*b* minus o_3" reference spectra, in spite of the fact that the heme composition of this enzyme is predominantly (about 70%) oo_3 (Puustinen et. al., 1992). This suggests that there is relatively more electron redistribution in the bo_3 subpopulation than in the oo_3 subpopulation, which would be consistent with the smaller

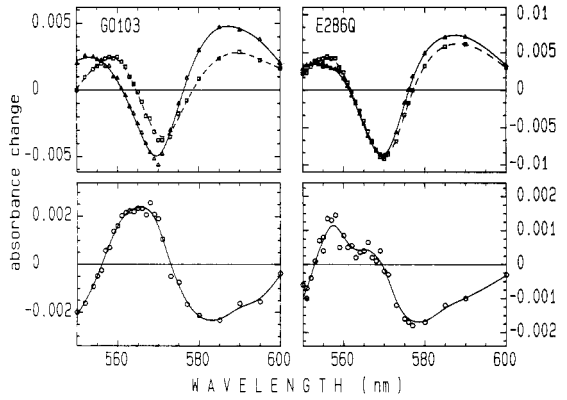

FIGURE 7: Spectra of the "unresolved" fast phase showing differences between COMV and COFR cytochrome *o* from the GO103 and E286Q strains. The top two panels show spectra for the COMV (- -□- -) and COFR (–△–) enzymes. The bottom two panels show the difference between the COMV and COFR spectra. Note that only the α-band region is shown. Amplitudes reflect the difference in absorbance levels before and after the jump which accompanies photolysis, as resolved on a microsecond time scale (see Figure 5): cytochrome *o* from GO103, 2.0 μM; from E286Q, 3.4 μM (for other conditions, see Figure 2).

net extent of electron transfer in the E286Q enzyme which we inferred from recombination rate data in Figure 2, and suggests that the redox potential of the low-spin heme B is higher than that of the low-spin heme O (see Discussion). In the RG145 enzyme, which is approximately half bo_3 and half oo_3, the fast phase spectra (not shown) look almost exactly like the spectra for the GO103 enzyme. This indicates that most electron transfer in this enzyme takes place in the bo_3 subpopulation and that the oo_3 electron redisribution can only be seen in the spectrum when the population of bo_3 is very small relative to that of oo_3, as is the case in E286Q.

The next phase which is observed at 390 nm (Figure 5) has a time constant of approximately 100 μs. The kinetic spectrum of this phase in the mixed-valence GO103 enzyme is very similar to the corresponding fast-phase spectra described above. This finding suggests that electron redistribution between the hemes of cytochrome *o* can have more than one characteristic rate. In fact, our data indicate that this electron redistribution may take place over an even wider range of time scales. The 390-nm kinetic traces (Figure 5) show two other clear differences between the reduced and the mixed valence enzymes, one seen in the first part of the 10-ms sweep and the other in the fast unresolved step where the photolysis occurs. However, in both cases the kinetic spectrum includes a contribution from some other process or processes in addition to the putative electron transfer.

In the case of the slower (millisecond) phase, it is difficult to construct a spectrum of the phase because there are absorbance changes on approximately the time scale in the fully reduced enzyme, which may or may not be present in the mixed-valence enzyme.

In the case of the fastest change, our instruments cannot resolve kinetic phases but can only measure the amplitude of the absorbance changes which occur. The spectra of this unresolved change in mixed-valence and fully reduced forms of the enzyme are shown in Figure 7 for the GO103 and E286Q strains. The spectra for COMV and COFR enzymes are clearly different, confirming that the difference in amplitudes at 390 nm (Figure 5) is not simply due to a difference in the efficiency of photolysis. The difference spectra (COMV minus COFR) in the lower panels of Figure 7 have features which are clearly indicative of electron transfer. In the case of the E286Q enzyme, the spectrum (lower right panel) has a sharp maximum near 556 nm, as does the corresponding reference spectrum in Figure 4. The spectrum for the GO103 enzyme (lower left panel) has a broad maximum centered near 565 nm which appears similar to the peak at 563 nm in the GO103 reference spectrum. The differences in both cases appear to come from an additional component which absorbs near 568 nm. As described above, if this is an electron-transfer process, we would expect the amplitude of the absorbance changes in the E286Q enzyme to be smaller than in the GO103 enzyme. This is indeed the case, as can be seen from Figure 7.

DISCUSSION

Photolysis of the CO-bound mixed-valence compound of cytochrome *c* oxidase is followed by fast electron redistribution between the enzyme's two hemes (Oliveberg & Malmström, 1991; Verkhovsky et al., 1992). We now report a corresponding electron-transfer process in cytochrome *o*. The rates we find for this process ($3 < \tau < 12$ μs) are comparable to the rates in cytochrome aa_3 ($\tau = 3$ μs). However, we also find that this microsecond process accounts for only a part of the total electron redistribution and that there appear to be phases of this process which take place on other time scales, both slower and faster.

We have also studied the influence of this electron redistribution on the rate with which CO recombines with the enzyme. As illustrated in Figure 1, fast electron reequilibration to the low-spin heme has the result that in a given molecule of the mixed-valence enzyme, after photolysis, the oxygen-binding heme is only partially reduced in the time-average. Since the oxidized heme is unable to bind CO, the mixed-valence enzyme will exhibit a lower apparent rate constant for CO rebinding than the reduced form. Given the CO-recombination rates for both the reduced and the mixed-valence forms of the enzyme, it is possible to calculate the equilibrium constant for electron distribution between the two hemes, $[b^{3+}o_3^{2+}]/[b^{2+}o_3^{3+}]$ (see Figure 1). The values obtained in this way vary slightly with enzyme preparation, but for cytochrome bo_3 (GO103 strain) the values generally lie between 1.5 and 2.5, which corresponds to a redox potential difference between the two hemes of 11–24 mV.

One interesting feature of cytochrome *o* is that the nature of the heme at the low-spin site is variable. Some strains incorporate almost exclusively heme B, while others incorporate heme O to varying extents (Puustinen et al., 1992). There is some evidence that the two kinds of heme in this site have different redox potentials (Bolgiano et al., 1991; Puustinen et al., 1992). A study of the recombination rates in the COMV forms of the enzyme from different strains showed that the more heme B is present at the low-spin heme site, the larger the extent of electron redistribution to that site, clearly indicating that heme B has a higher potential than heme O. This relationship could also be seen in the extents of the electron transfer phases themselves. These extents also allow us to estimate the redox potential difference beween hemes B and O at the low potential site: Assuming that the E286Q enzyme contains 70% heme *o* at the low-spin site and that the equilibrium constant for the bo_3 enzyme for the GO103 strain is 2 (this is the average of the above values), the equilibrium constant $[o^{3+}o_3^{2+}]/[o^{2+}o_3^{3+}]$ would be 6, and the redox potential difference would be 50 mV.

There are, however, strong indications that the electron exchange between hemes o_3 and *b* is slightly faster than between hemes o_3 and *o*, at least for the microsecond phase; the wild type enzyme, which is a cytochrome bo_3, gives almost exactly the same rate for the microsecond phase as is found in bovine aa_3, whereas the oo_3 enzyme gives a somewhat slower rate. This difference is likely to arise from the steric properties

of the two kinds of heme rather than energetic factors. Given the redox potentials estimated above, Marcus theory (Marcus & Sutin, 1985) predicts that the relaxation rate for the oo_3 system should be faster, rather than slower, since the driving force is larger[2] (the apparent rate here is the sum of the forward and the reverse rate constants, and the "forward" rate, with respect to enzyme function, will dominate). The biggest structural difference is that heme O has a long farnesylhydroxyethyl chain attached to its edge (Puustinen & Wikström, 1991; Wu et al., 1992). Incorporating this structure in a site where it does not fit correctly may change the position of the heme and thus alter the distance of the electron transfer, or some other relevant parameter.

It appears that, in cytochrome *o*, this electron redistribution process takes place with three or four different rates. The kinetic spectra for these different phases are similar, suggesting that they all reflect the same net process, flow of electrons from the oxygen-binding heme to the low-spin heme, and that they are not sequential reactions. The first two phases appear to be authentic electron transfer kinetic processes, i.e., where the electron tunneling per se is rate limiting. In the case of the microsecond phase this is indicated by the fact that the rate of the process is temperature independent from 6 to 40 °C (data not shown). We could not study the temperature dependence of the rate of the unresolved phase, but the speed of the process and the fact that it is faster than a phase which has been shown to be independent of temperature both argue that here, too, electron transfer is rate limiting. One possible explanation for these two rates could be that they arise from two different states of the oxygen-binding site as it relaxes in the first few microseconds following photolysis of CO (Findsen et al., 1987).

The slower phases (hundreds of microseconds to milliseconds) could arise from states of the enzyme where the physical process of inter-heme electron transfer is inherently slower. In this case, the observed rate could be either the electron-transfer rate constant or a rate of escape into a state of the enzyme where electron transfer is more facile. Alternatively, these phases could arise from slow relaxation processes in the enzyme which realign the redox levels of the two hemes after CO photolysis. Photolysis immediately lowers the redox potential of the oxygen-binding heme, but there could be subsequent relaxations which would drop the potential further, and with a slower rate. Examples of this might include a proton uptake/release event or a conformational relaxation.

The possibility exists that these slow phases are artifactual and represent a subpopulation of enzyme which has been damaged during the isolation procedure. Provided that the unresolved phase actually includes an electron transfer, we can conclude that the largest part of the electron-transfer process takes place on a microsecond time scale or faster. Thus, if these slower phases arise from damaged enzyme, the size of this damaged subpopulation would be quite small.

We cannot rule out the possibility that the fastest (unresolved) phase arises from a spectral interaction between the two hemes rather than from a true electron transfer. Two kinds of interaction might be invoked to explain this: (1) The spectrum of the photolysis event could be sensitive to the nature of the heme (B or O) at the low-spin site. It is difficult to imagine, however, how such an interaction could produce spectral features that look like the redox spectra of the appropriate low-spin heme. (2) The spectrum of the low-spin heme could be altered (in amplitude or shape) by the photolysis of CO bound at the other heme. If this were the case, the largest differences in photolysis spectra between bo_3 and oo_3 enzymes would be expected between the reduced forms, since the putative interaction spectra show strong contributions from the reduced spectra. However, the photolysis spectra for the reduced forms for different types of enzyme are almost identical, while photolysis spectra for the mixed-valence enzymes are strikingly different. Thus, although we cannot rule out an explanation which does not involve electron transfer, other explanations appear unlikely. At present, work is in progress to resolve this phase, in order to answer this question directly.

[2] Under conditions of high driving force, it is possible for an increase in the driving force of an electron-transfer process to lead to a decrease in the rate. However, the driving forces of the processes under consideration here are much too small for such "inverted region" effects to be observed (Marcus and Sutin, 1985).

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