A New Ruthenium Complex To Study Single-Electron Reduction of the Pulsed O_H State of Detergent-Solubilized Cytochrome Oxidase†

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ABSTRACT: The first step in the catalytic cycle of cytochrome oxidase, the one-electron reduction of the fully oxidized enzyme, was investigated using a new photoactive binuclear ruthenium complex, [Ru-(bipyrazine)$_2$]$_2$(quaterpyridine), (Ru_2Z). The aim of the work was to examine differences in the redox kinetics resulting from pulsing the oxidase (i.e., fully reducing the enzyme followed by reoxidation) just prior to photoreduction. Recent reports indicate transient changes in the redox behavior of the metal centers upon pulsing. The new photoreductant has a large quantum yield, allowing the kinetics data to be acquired in a single flash. The net charge of +4 on Ru_2Z allows it to bind electrostatically near Cu_A in subunit II of cytochrome oxidase. The photoexcited state Ru(II*) of Ru_2Z is reduced to Ru(I) by the sacrificial electron donor aniline, and Ru(I) then reduces Cu_A with yields up to 60%. A stopped-flow-flash technique was used to form the pulsed state of cytochrome oxidase (the "O_H" state) from several sources (bovine heart mitochondria, *Rhodobacter sphaeroides*, and *Paracoccus denitrificans*). Upon mixing the fully reduced anaerobic enzyme with oxygenated buffer containing Ru_2Z, the oxidized O_H state was formed within 5 ms. Ru_2Z was then excited with a laser flash to inject one electron into Cu_A. Electron transfer from Cu_A → heme *a* → heme a_3/Cu_B was monitored by optical spectroscopy, and the results were compared with the enzyme that had not been pulsed to the O_H state. Pulsing had a significant effect in the case of the bovine oxidase, but this was not observed with the bacterial oxidases. Electron transfer from Cu_A to heme *a* occurred with a rate constant of 20 000 s^{-1} with the bovine cytochrome oxidase, regardless of whether the enzyme had been pulsed. However, electron transfer from heme *a* to the heme a_3/Cu_B center in the pulsed form was 63% complete and occurred with biphasic kinetics with rate constants of 750 s^{-1} and 110 s^{-1} and relative amplitudes of 25% and 75%. In contrast, one-electron injection into the nonpulsed O form of the bovine oxidase was only 30% complete and occurred with monophasic kinetics with a rate constant of 90 s^{-1}. This is the first indication of a difference between the fast form of the bovine oxidase and the pulsed O_H form. No reduction of heme a_3 is observed, indicating that Cu_B is the initial electron acceptor in the one-electron reduced pulsed bovine oxidase.

Cytochrome *c* oxidase is a redox-linked proton pump which uses four electrons from cytochrome *c* to reduce molecular oxygen to water (*1*, *2*). Electron transfer is coupled to the uptake of four "chemical" protons from the matrix to combine with O_2 to form $2H_2O$ and the translocation of four additional "pumped" protons from the matrix to the cytoplasmic side of the membrane (*3*, *4*). The reaction begins with reduction of Cu_A by cytochrome *c*, followed by electron transfer from Cu_A to heme *a*, and then to the heme a_3−Cu_B binuclear center (*5*−*9*). The enzyme with a fully oxidized heme a_3/Cu_B center, state O, is reduced in two successive one-electron-transfer steps to form state E and then state R_2 (Figure 1). Molecular oxygen rapidly binds to state R_2 and

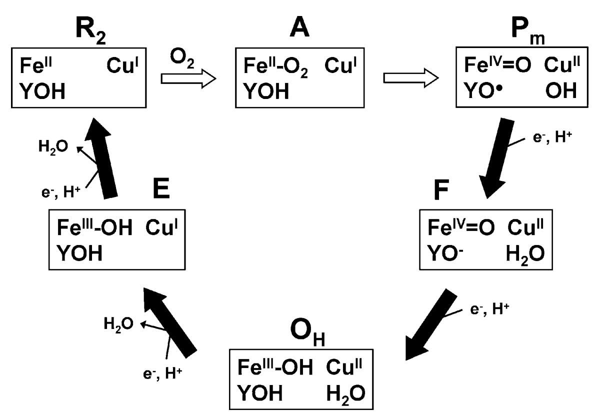

FIGURE 1: Catalytic cycle of cytochrome oxidase. The states of the binuclear center heme a_3, Cu_B, and the conserved tyrosine are shown. Reaction steps indicated by solid arrows are coupled to proton pumping.

is reduced in a rapid four-electron reaction to form state P_M, in which the electrons come from heme a_3 (Fe^{+2} → Fe^{+4} =

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O^{-2}), Cu_B ($Cu^{+1} \rightarrow Cu^{+2}OH^{-1}$) and from a nearby amino acid, probably Y244(bovine) (TyrOH → TyrO•) (*10*, *11*). In successive one-electron-transfer reactions, the tyrosine radical in state P_M is reduced, forming state F, and then the oxyferryl heme a_3 is reduced to form ferric heme a_3 in state O.

There is growing experimental support for models in which each of the one-electron transfers to the heme a_3/Cu_B center in the oxidase is coupled to pumping one proton across the membrane (*12*−*18*). Evidence for proton pumping coupled to individual steps is best documented for the F → O and P_M → F transitions. In the F → O transition, electron transfer from heme *a* to oxyferryl heme a_3 has a rate constant of about 660 s^{-1} in the bovine enzyme, whereas coupled proton transfer, deduced from the resulting generation of a transmembrane voltage, is biphasic with rate constants of 830 s^{-1} and 220 s^{-1} and relative amplitudes of 1:3 (*19*−*23*). In the P_M → F transition, electron transfer from heme *a* to (presumably) the Tyr 244 radical has a rate constant of 4000 s^{-1}, while coupled proton transfer is triphasic, with rate constants of 3300 s^{-1}, 770 s^{-1}, and 150 s^{-1} (relative amplitudes 1:1.3:0.5) (*24*, *25*). Thus, in both the P_M → F and F → O transitions, a large part of coupled proton transfer occurs after electron transfer is complete.

If an electron is already present on heme *a* at the time of the reaction with O_2, as is the case when the fully reduced enzyme is reacted with O_2, the initial product is the P_R state, in which an electron from heme *a* is used to reduce O_2 in place of the electron from the active-site tyrosine. The subsequent P_R → F and F → O steps have also been demonstrated to pump one proton each (*26*, *27*).

The evidence for proton pumping coupled to the O → E (*28*, *29*) and E → R_2 (*30*) transitions is less clear because the reduction kinetics depends on the way in which the oxidized enzyme has been prepared prior to the experiment. A large number of different forms of the oxidized enzyme have been operationally described in the literature, including "resting" (*31*, *32*), "slow" (*33*−*38*), "fast" (*34*, *36*, *38*), "pulsed" (*31*, *32*), O (*38*−*40*), and O_H (or H) (*38*−*40*) states. Purification of the bovine oxidase can yield enzyme that is either in the "resting" or "fast" form or a mixture. The "resting" enzyme is characterized by a slow catalytic rate due to slow electron transfer to the heme a_3/Cu_B center. The "slow" form of the oxidase can be generated by incubation of the enzyme at pH 6.5, and this form of the enzyme appears equivalent to the resting form; i.e., the enzyme binds to cyanide slowly, has a slow rate of electron transfer from heme *a* to the heme a_3/Cu_B center, has a blue-shifted Soret band, and has a prominent $g = 12$ EPR signal (*37*). The molecular causes for most of the differences between the various forms are not known, but the presence of either endogenous or exogenous anionic ligands to the heme a_3/Cu_B metal centers is certainly one important factor (*37*).

The rate of electron transfer from Cu_A to heme *a* is similar for all forms of the oxidized enzyme, but the rate of electron transfer from heme *a* to the heme a_3/Cu_B center varies over orders of magnitude depending on how the enzyme has been handled. The "slow" oxidase can be activated to the "pulsed" form by complete reduction followed by reaction with O_2. In time, the pulsed form of the oxidase will decay back to the slow form, at a rate depending on solution conditions. Purification protocols for the bovine enzyme have been developed that yield an enzyme that is in the "fast" form and has characteristics equivalent to the "pulsed" oxidase (*38*).

Recent work from the Wikström laboratory (*28*, *29*, *39*, *40*) has demonstrated that proton pumping coupled to the reductive steps in the catalytic cycle (the O → E and E → R_2 transitions) is only observed if the enzyme is reduced very soon after pulsing. This state has been called the O_H (or H) state of the enzyme. This has been most clearly demonstrated in work with the cytochrome oxidase from *Paracoccus denitrificans* (*28*, *29*, *39*, *40*). It was shown that the one-electron reduction of the O_H form of the oxidase initiates a sequence of cascading equilibria of proton and electron transfers, ending up with the electron residing entirely on Cu_B and one proton being pumped. The data indicate that formation of the pulsed O_H state transiently results in an increase of the midpoint potential of Cu_B at least 100 mV higher than that of heme *a*. Thus, electron transfer from heme *a* to the heme a_3/Cu_B center is complete within 2 ms and with a sufficient driving force to pump a proton across the membrane. This transient O_H form of the oxidase would be expected to be very unstable and, thus, distinct from the "fast" form of the enzyme, which is stable.

Recently, the spectroscopic and kinetics properties of the "activated" (O_H) form of the bovine oxidase were compared to those of the isolated enzyme in the "fast" form (*38*). It was determined that the properties immediately after pulsing were indistinguishable from those measured in the absence of the pulsing procedure. Specifically, the rate of reduction of the heme a_3/Cu_B center was determined using stopped-flow spectroscopy by mixing the enzyme with ruthenium hexamine, a relatively strong reductant. No differences were observed between the O_H and "fast" forms of the enzyme.

In the current work, a new photoactivated reductant, Ru_2Z,[1] is used to rapidly inject one electron into the pulsed (O_H) or nonpulsed ("fast") forms of the bovine oxidase. Remarkably, and in contrast to the results obtained with ruthenium hexamine, both the rate and extent of electron transfer from heme *a* to the heme a_3/Cu_B center are significantly increased by the pulsing procedure. The data demonstrate that there is a difference in the properties of the bovine oxidase between the O_H and "fast" forms. Extensive efforts to demonstrate the same effect with the prokaryotic oxidases from *Rhodobacter sphaeroides* and from *P. denitrificans*. were not successful.

MATERIALS AND METHODS

Materials. L-Ascorbic acid, cardiolipin (C5646), L-α-phasphatidylcholine (P3644), lauryl maltoside (LM), aniline, 3CP, $RuCl_3 \cdot nH_2O$, and phenazine methosulfate (PMS) were obtained from Sigma Aldrich Co. The preparation of the K13E mutant of horse cytochrome *c* will be described elsewhere (Davis et al., in preparation).

Synthesis of Ru_2Z. Ru_2Z was synthesized as described in Figure 2. 2,2′-Bipyrazine (bpz) was prepared by the method of Rillema et al. (*41*). $Ru(bpz)_2Cl_2$ was prepared from 2,2′-bipyrazine and $RuCl_3 \cdot nH_2O$ according to literature methods

[1] Abbreviations: Ru_2Z or $[Ru(bpz)_2]_2$(qpy), $[Ru(bipyrazine)_2]_2$-(quaterpyridine); bpz, 2,2′-bipyrazine; qpy, 2,2′:4′,4″:2″,2‴-quaterpyridine; Ru_2C, $[Ru(bipyridine)_2]_2$(1,4-bis[2-(4′-methyl-2,2′-bipyrid-4-yl)ethenyl]benzene)$(PF_6)_4$; $Ru(bpy)_3$, $Ru(bipyridine)_3$; 3-CP, 3-carboxy-2,2,5,5-tetramethyl-1-pyrrolidinyloxy free radical; LM, lauryl maltoside.

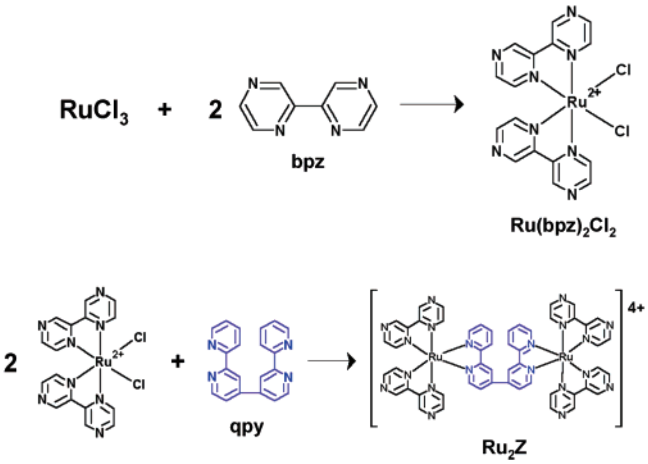

FIGURE 2: Synthesis of Ru_2Z.

Table 1: Standard Reduction Potentials of Ruthenium Complexes vs Normal Hydrogen Electrode

complex	(II)/(III)	(II*)/(III)	(II)/(I)	(II*)/(I)
Ru_2C	1.27	−0.87	−1.31	0.83
Ru_2Z	1.7	−0.28	−0.82	+1.16

(*42*). 2,2′:4′,4′′:2′′,2′′′-quaterpyridine (qpy) was prepared by the method of Downard et al., (*43*). Ru_2Z was prepared by refluxing 0.017 g (0.055 mmol) of qpy and 2 equiv of Ru(bpz)$_2$Cl$_2$ (0.10 g, 0.2 mmol) in 20 mL of ethylene glycol under nitrogen for 12 h. The product was allowed to cool and was collected as the PF_6 salt by adding an excess amount of NH_4PF_6 dissolved in water. The solid was then recrystallized from acetonitrile and purified through size exclusion chromatography on a Sephadex LH-20 column with dichloromethane (CH_2Cl_2) and an increasing volume of acetonitrile as the eluent. The pure product eluted with CH_2Cl_2/acetonitrile (1:1). The final product was recrystallized from acetonitrile and characterized to give UV (H_2O) 458 nm and ^{1}H NMR (270 MHz, ACN-d3) $\delta = 7.53$ (t, $J = 6.3$ Hz, 2H), 7.75 (d, $J = 5.1$ Hz, 2H), 7.81 (m, 4H), 7.88 (t, $J = 6.2$ Hz, 8H), 8.21 (t, $J = 7.8$ Hz, 2H), 8.61 (d, $J = 3.2$ Hz, 8H), 8.76 (d, $J = 7.9$ Hz, 2H), 8.87 (s, 2H), 9.78 (s, 8H).

Spectroscopic Measurements. Visible/UV spectra were obtained with a Hewlett-Packard model 8452A diode array spectrophotometer. NMR characterization was done on a 270 MHz Jeol EX270 ^{1}H-NMR spectrometer. The reduction potentials of Ru_2Z were determined by cyclic voltammetry using a custom built potentiometer interfaced to a PC. The sample was dissolved in acetonitrile with 0.1 M tetrabutyl ammonium PF_6 as the supporting electrolyte with a Pt-bead working electrode and a Ag/AgCl reference electrode. The sample was purged with nitrogen prior to measurement. The ground state reduction potentials of Ru_2Z were found to be E(II/III) = +1.7 V and E(I/II) = −0.82 V (Table 1). E(II/III) and E(I/II) were subtracted from E_{00}(II*) to give E(II*/III) of −0.28 V and E(II*/I) of +1.16 V, respectively. The value of E_{00}(II*) for Ru_2Z was determined to be +1.98 V from fluorescent measurements in a 4:1 ethanol:methanol solution at 77 K using a Hitachi model F2500 fluorescent spectrophotometer. Excited-state lifetime measurements were obtained from a room temperature aqueous solution in a 1 cm quartz microcuvette with a 355 nm QuantaRay DCR-1 Nd:YAG laser having a 10 ns pulse width as a light source. The emitted radiation was monitored at right angles to the incident beam by a photomultiplier tube.

Cytochrome Oxidase Preparations. "Fast" bovine cytochrome oxidase was prepared as described in reference 38. This oxidase preparation was characterized by studying its reduction by ruthenium hexamine and dithionite as described by Jancura et al. (*38*). More than 80% of the heme a_3 was reduced in less than 1 s, indicating that the preparation was 80% fast. In another criteria for the fraction of fast oxidase, 70% of the oxidase reacted rapidly with H_2O_2, indicating that it was 70% fast. The turnover number of this preparation was 530 ± 100 s^{-1}. *R. sphaeroides* cytochrome oxidase preparation A was purified as described by Mitchell et al. (*44*). Preparation B was purified by the same method except that a lower concentration of LM was used to minimize loss of lipids. The membranes were solubilized in 0.8% LM for 45 min, and the chromatography steps were carried out in 0.05% LM. Preparation C was obtained by enriching preparation B with cardiolipin using the procedure of Lee et al. (*45*). Dried cardiolipin (Sigma C5646 from bovine heart, 80% polyunsaturated) was added to a buffer solution consisting of 0.5 mM ATP, 10 mM K-Hepes pH 7.4, 40 mM KCl, and 1% Tween 20. The oxidase was added in a 200:5 (μmol:μmol) ratio of cardiolipin to enzyme and incubated for 1 h at 4 °C. The oxidase was equilibrated twice with the buffer used for kinetic experiments using a Centricon-100 concentrator. Preparation D was obtained using the same procedure as preparation C with 1 mg/mL sonicated phosphatidyl choline (Sigma P3644 from soybean) included in the incubation buffer. *P. denitrificans* cytochrome oxidase was a generous gift from Bernd Ludwig (Institute of Biochemistry, and Institute of Biophysical Chemistry, Frankfurt, Germany). The steady-state turnover number was 750 ± 250 s^{-1}.

Steady-State Activity. The steady-state maximal turnover numbers of the oxidase preparations were measured spectroscopically with 50 μM reduced horse heart cytochrome *c* in 50 mM sodium phosphate, pH 6.0, at 25 °C. At least three measurements were made for each sample, and the error given is the standard deviation.

Stopped-Flow-Flash Photolysis. A Hi-Tech SF-61 stopped-flow apparatus was used to rapidly mix reduced, anaerobic oxidase with oxygenated buffer containing the Ru_2Z complex. The dimensions of the fused UV silica optical cell are 10 × 1.5 × 1.5 mm, with the probe beam passing through the 10 mm path, and the pulse beam through the 1.5 mm path. The probe beam system has been previously described (*22*). The sample was excited 5−100 ms after stopped-flow mixing with a Phase R Model DL 1400 flash lamp-pumped dye laser using coumarin 480 to produce a 480 nm laser flash with a duration of <0.5 μs. To prepare the oxidase sample, the enzyme solution was first purged with argon to completely remove atmospheric oxygen, and then the oxidase was reduced with 4 mM ascorbate and 2 μM PMS. Complete reduction of both hemes *a* and a_3 were observed in the HP spectrophotometer within 2 min. The sample was then transferred under anaerobic conditions to one syringe of the stopped-flow instrument, and it was allowed to sit for at least 15 min to ensure complete reduction. An oxygenated buffer solution containing Ru_2Z, aniline, and 3CP was added to the other syringe, and the stopped-flow-flash experiment was initiated. Final reaction solutions typically contained 20 μM

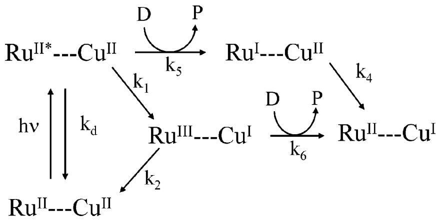

FIGURE 3: Photoreduction of Cu_A in cytochrome oxidase by Ru_2Z. D is the sacrificial electron donor aniline, and P is its oxidation product.

Ru_2Z, 5 μM oxidase, 2 mM ascorbate, 1 μM PMS, 10 mM aniline, and 1 mM 3CP in 5 mM HEPES buffer, pH 8.0, 0.1% LM. Catalase and superoxide dismutase were present in the buffer to react with any hydrogen peroxide or superoxide produced. Aniline and carboxyl-2,2,5,5-tetramethyl-1-pyrolidinyloxy free radical (3CP) were used as sacrificial electron donors to the ruthenium complex. Reduction of Cu_A was measured at 830 nm with $\Delta\epsilon = 2.0$ mM^{-1} cm^{-1}. Reduction of heme *a* was measured at 605 nm using $\Delta\epsilon = 16$ mM^{-1} cm^{-1}. Each transient was recorded for a single laser flash with no signal averaging. Absorbance transients were fitted as described in Zaslavaky et al. (*22*). At least three independent measurements were made for each set of conditions, and the error given for the rate constant is the standard deviation.

RESULTS

Synthesis and Characterization of Ru_2Z. The binuclear ruthenium complex Ru_2Z was prepared as shown in Figure 2. It has a UV/visible absorption spectrum with a maximum at 458 nm in water with an extinction coefficient of 21 000 M^{-1} cm^{-1}. Photoexcitation of Ru_2Z produces a metal-to-ligand charge transfer (MLCT) state of the ruthenium complex, which has a broad luminescence band between 600 and 650 nm at 77 K, in 4:1 ethanol/methanol. The emission maximum is a single peak at 626 nm that translates to an $E_{00}(2+^*)$ of +1.98 V. Emission lifetime measurement in H_2O showed an MLCT state with a lifetime of 0.4 μs at room temperature. The redox potentials of Ru_2Z were obtained with cyclic voltammetry to give *E*(II/III) of +1.7 V and *E*(I/II) of −0.82 V (Table 1). *E*(II/III) and *E*(I/II) were subtracted from E_{00}(II*) to give *E*(II*/III) of −0.28 V and *E*(II*/I) of +1.16 V, respectively.

The binuclear ruthenium complex, Ru_2Z, was found to be efficient in the photoreduction of oxidase (Figure 3). Flash photolysis of a sample containing 20 μM Ru_2Z and 5 μM bovine oxidase in 5 mM sodium phosphate, pH 7.0, 10 mM aniline, and 1 mM 3CP led to rapid photoreduction of Cu_A, followed by electron-transfer equilibrium with heme *a* with a rate constant of 20 000 s^{-1}. After rapid equilibrium, 75% of the electron resided on heme *a* and 25% on Cu_A. The total yield of photoreduced Cu_A/heme *a* was 60% following a single flash under aerobic conditions and even higher under anaerobic conditions. By comparison, the photoreduction yields for Ru_2C and $Ru(bpy)_3$ were 10% and 2% under similar aerobic conditions (*22*). The larger yield for Ru_2Z is due to the high redox potential of +1.16 V for the Ru(II*)/Ru(I) reaction (Table 1), allowing the sacrificial electron donor aniline to directly reduce the excited state Ru(II*) to Ru(I), which then rapidly transfers an electron to Cu_A (Figure 3). Oxygen also reacts with Ru(I), accounting for the smaller yield of oxidase photoreduction under aerobic conditions. The decay of Ru(I) under aerobic conditions has a rate constant of 80 000 s^{-1}. Ru_2Z, thus, photoreduces Cu_A in oxidase using the k_5 and k_4 reactions shown in Figure 3. By comparison, the redox potential for the Ru(II*)/Ru(I) reaction of Ru_2C is only +0.83 V, which is insufficient for aniline to reduce Ru(II*), so the reduction of Cu_A by Ru_2C goes by the k_1 and k_6 pathways.

One-Electron Reduction of Bovine Oxidase in the O and O_H States. A stopped-flow-flash technique was used to study the one-electron reduction of pulsed bovine oxidase in the O_H state. The enzyme was prepared using a protocol yielding bovine oxidase that was 70−80% in the "fast" form (*38*). Oxidase (5.0 μM final concentration) was anaerobically reduced in one syringe of the stopped flow with 2 mM ascorbate and 1 μM PMS in 20 mM HEPES buffer, pH 8.0, and then mixed with oxygenated buffer. Heme a_3 was 85% reoxidized after 20 ms compared to the original fully oxidized enzyme, indicating that 15% of heme a_3 did not react rapidly with oxygen. To study one-electron reduction of the O_H state, fully oxidized oxidase (5.0 μM final concentration) was anaerobically reduced in one syringe of the stopped flow with 2 mM ascorbate and 1 μM PMS in 5 mM HEPES buffer, pH 7.9, and mixed with oxygenated buffer containing 20 μM Ru_2Z, 10 mM aniline, and 1 mM 3CP. The sample in the stopped-flow cell was subjected to a 480 nm laser flash 20 ms after mixing to photoexcite Ru_2Z to the Ru(I) state, which injected a single electron into Cu_A. The absorbance transient at 830 nm indicated that Cu_A was rapidly reduced and then reoxidized with a rate constant of 20 000 ± 3000 s^{-1} due to electron transfer to heme *a* (Figure 4A). The reduction of heme *a*, with a rate constant of 20 000 ± 3000 s^{-1}, was observed at 605 nm (Figure 4A). Rapid equilibrium is established between Cu_A and heme *a* by 0.3 ms, at which point heme *a* is 52% reduced and Cu_A is 16% reduced, giving an equilibrium constant *K* for electron transfer from Cu_A to heme *a* of 3.2, indicating that the redox potential of heme *a* is 30 mV more positive than that of Cu_A at this point in the reaction. Biphasic reoxidation of heme *a*/Cu_A was observed at a longer time scale, with rate constants of 750 ± 100 s^{-1} and 110 ± 20 s^{-1} and relative amplitudes of 25% and 75% (Figure 4B). The total reoxidation of heme *a* was 63%. Reduction of heme a_3 was not observed at 436 nm, indicating that the electron acceptor in the binuclear site is Cu_B and not heme a_3 (Figure 4B). No reaction was observed at 580 nm, demonstrating state F was not involved (Figure 4B). All the transients were for a single flash with no signal averaging, and they were independent of the delay between mixing and flashing over the range 5 to 100 ms. To examine single-electron reduction of the resting O state of the bovine oxidase, the fully oxidized enzyme, previously characterized as being 70−80% in the "fast" form, was placed in one of the stopped-flow syringes in aerobic 5 mM HEPES buffer, pH 7.9. This was mixed with a solution of the same aerobic buffer containing 20 μM Ru_2Z, 10 mM aniline, and 1 mM 3CP, and then excited with a laser flash to inject one electron into Cu_A. Following electron transfer from Cu_A to heme *a* with a rate constant of 20 000 s^{-1}, monophasic reoxidation of heme *a* occurred with a rate constant of 90 ± 20 s^{-1} and an extent of 30% (Figure 4C). Precautions were taken to keep the sample in the dark before the flash, and catalase and superoxide dismutase were

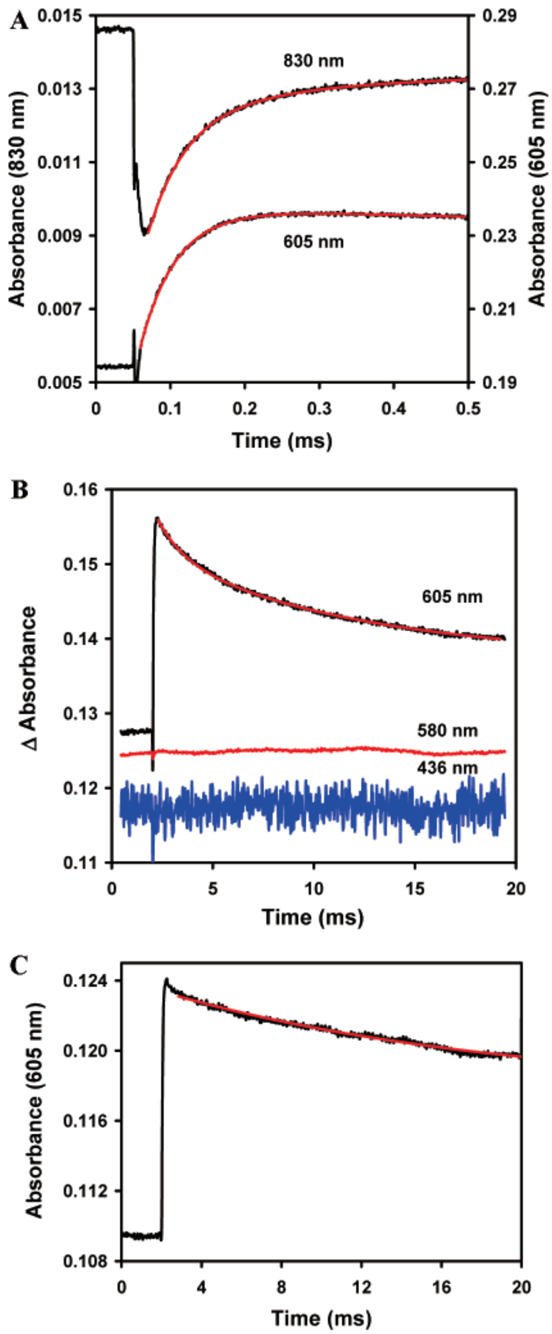

FIGURE 4: Photoinjection of one electron into the O_H form of bovine oxidase. Reduced oxidase (5.0 μM final concentration in 2 mM ascorbate, 1 μM PMS, 5 mM HEPES, pH 7.9, and 0.1% LM) was mixed in the stopped-flow cell with oxygenated buffer containing 20 μM Ru_2Z, 10 mM aniline, and 1 mM 3CP to form the O_H state, and it was excited with a laser flash after 20 ms to inject an electron into Cu_A. A: The 830 and 605 nm absorbance transients indicate rapid reduction of Cu_A followed by electron transfer from Cu_A to heme *a* with a rate constant of 20 000 ± 3000 s^{-1}. B: The 605 nm transient at longer times shows biphasic reoxidation of heme *a* with rate constants of 750 and 110 s^{-1}. There were no changes in the 436 or 580 nm absorbance transients. C: One-electron injection into the nonpulsed O state of fast bovine oxidase. The 605 nm trace shows the reduction of resting heme *a* with a rate constant of 20 000 s^{-1} and monophasic reoxidation with a rate constant of 90 s^{-1} and an extent of 30%.

included in the buffer to destroy any hydrogen peroxide or superoxide that might have been formed.

One-Electron Reduction of Bacterial Oxidases in the O and O_H States. The injection of one electron into the "as isolated" O state and into the pulsed O_H state of the *R. sphaeroides* oxidase was studied using the same technique described above for bovine oxidase. Rapid photoreduction of Cu_A in the nonpulsed O state of *R. sphaeroides* oxidase preparation A led to electron transfer from Cu_A to heme *a* with a rate constant of 90 000 ± 20 000 s^{-1}, followed by monophasic reoxidation of heme *a* with a rate constant of 1000 ± 200 s^{-1} (Table 2). The extent of reoxidation of heme *a* was only 25%. Photoreduction of the pulsed O_H state of preparation A resulted in electron transfer from Cu_A to heme *a* with a rate constant of 90 000 s^{-1}, followed by monophasic reoxidation of heme *a* with a rate constant of 760 ± 200 s^{-1} and an extent of 26% (Table 2). Hence, pulsing the *R. sphaeroides* oxidase neither increases the extent of heme *a* reoxidation nor speeds up electron transfer to the heme a_3/Cu_B center.

Table 2: Photoinjection of One Electron into Nonpulsed and Pulsed Forms of *R. sphaeroides* Cytochrome Oxidase[a]

prep	state	TN (s^{-1})	k_2 (s^{-1})	% reox
A	nonpulsed	1500 ± 200	1000 ± 200	25 ± 5
A	pulsed	1500 ± 200	760 ± 200	26 ± 5
B	pulsed	1400 ± 200	740 ± 200	30 ± 5
C	pulsed	1550 ± 200	1500 ± 300	27 ± 5
C	nonpulsed	1550 ± 200	1800 ± 300	32 ± 5
D	pulsed	1600 ± 200	1350 ± 250	37 ± 6

[a] The kinetic studies were carried out as described in the text. The rate constant for reoxidation of heme *a* is k_2, The percent reoxidation of heme *a* is given under % reox. Preparations A−D were obtained as described in the Materials and Methods section. The steady-state turnover numbers, TN, were measured as described in the Materials and Methods section.

The kinetics of a number of different preparations of *R. sphaeroides* oxidase were examined (Table 2). Preparation A used in the experiments described above was prepared by the histidine tagged affinity chromatography method of Mitchell et al. (*44*). Essentially the same kinetic results were observed in preparation B which was purified using a lower LM concentration to minimize loss of phospholipids (Table 2). The rate constant for reoxidation of heme *a* was 1500 ± 300 for the pulsed form of preparation C incubated with cardiolipin, somewhat larger than for preparations A and B (Table 2). However, the extent of reoxidation was only 27%, nearly the same as for the nonpulsed form of preparation C. Similar kinetics were observed for preparation D incubated with both cardiolipin and phosphatidyl choline (Table 2).

The reduction of the O_H state of the *P. denitrificans* oxidase was also examined using the same technique described above. Following electron transfer from Cu_A to heme *a*, with a rate constant of 80 000 ± 15 000 s^{-1}, partial reoxidation of heme *a* was observed with a rate constant of 3000 ± 500 s^{-1} and reoxidation extent of 25%.

Injecting a Second Electron into the Bovine Oxidase. The one-electron photoreduction of the O_H state is meant to mimic the first step of the catalytic cycle as it would occur during steady-state turnover, the $O_H \rightarrow E$ transition. The one-electron reduction of the E state will form the two-electron reduced enzyme (R_2), which is expected to react rapidly with O_2 to form the P_M intermediate (*26*). The net reaction $E \rightarrow R_2 \rightarrow P_M$ was examined using Ru_2Z as the photoreductant in the following manner. The fast bovine oxidase (5 μM) and the K13E mutant of horse heart cytochrome *c* (5.3 μM) were

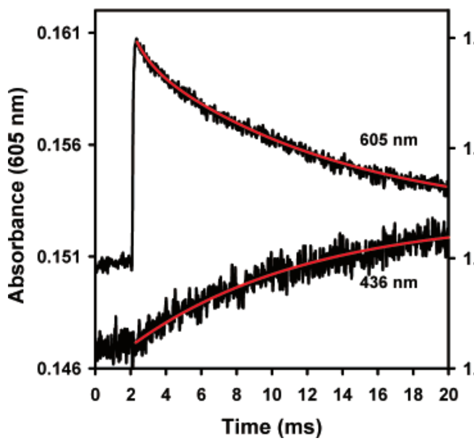

FIGURE 5: Injection of a second electron into pulsed O_H state of fast bovine oxidase. "Fast" bovine oxidase (5.0 μM final concentration) and 5.3 μM K13E horse cyt *c* were anaerobically reduced in one syringe of the Hi-Tech stopped-flow with 2 mM ascorbate and 1 μM PMS in 5 mM HEPES, pH 8.0, 0.1% LM. The sample was then mixed with oxygenated buffer containing 20 μM Ru_2Z, 10 mM aniline, and 1 mM 3CP. The oxidized O_H state formed within 1 ms was reduced to the E state within 5 ms. The sample was subjected to a 480 nm laser flash 20 ms after mixing to photoexcite Ru_2Z to the Ru(I) state, which injected a single electron into Cu_A. Biphasic reoxidation of heme *a* was observed at 605 nm with rate constants of 1100 s^{-1} and 90 s^{-1} and relative amplitudes of 11% and 89%. The 436 nm transient was consistent with the formation of state P with a rate constant of 90 s^{-1}.

anaerobically reduced in one syringe with 2 mM ascorbate and 1 μM PMS in buffer. The sample was then mixed in the stopped flow with oxygenated buffer containing 20 μM Ru_2Z, 10 mM aniline, and 1 mM 3CP. Upon mixing, the reduced enzyme is fully oxidized rapidly to the O_H state and is then reduced by the ferrous K13E cytochrome *c* within about 10 ms. Since the ratio of oxidase to cytochrome *c* is 1:1, the majority of the enzyme is reduced by one electron. The K13E cytochrome *c* mutant was used because it has a decreased binding affinity to the oxidase under the conditions used (*47*), so that after binding and donating one electron, the oxidized cytochrome *c* rapidly dissociates, allowing the Ru_2Z to bind to the same site on the oxidase. The laser was flashed 20 ms after mixing to photoreduce heme *a*/Cu_A. Biphasic reoxidation of heme *a*/Cu_A was observed with rate constants of 1100 s^{-1} and 90 s^{-1} and relative amplitudes of 11% and 89%. (Figure 5). The 436 nm transient was consistent with the formation of state P with a rate constant of 90 s^{-1} (Figure 5). Further photoreduction using multiple flashes led to a more rapid phase of reoxidation of heme *a* with a rate constant of 3000 s^{-1}. This rate is the same as previously measured for the $P_M \rightarrow F$ transition (*25*). It is noted that there is no detectable 3000 s^{-1} phase of heme *a* reoxidation associated with the first laser flash. This indicates that an insignificant portion of the oxidase is reduced by two electrons by the cytochrome *c*, since the resulting P_M state, following the reaction with O_2, would be converted to the F state during the first laser flash, and this is not observed.

DISCUSSION

A New High Yield Photoreductant. Ruthenium complexes have been very useful for studies of electron transfer in metalloproteins (*20*−*25*, *45*−*46*, *48*−*50*). These studies have employed either ruthenium complexes covalently attached to a metalloprotein (*45*−*46*, *48*) or ruthenium complexes in solution which interact with the metalloprotein noncovalently (*20*−*25*, *50*). Nilsson first used $Ru(bpy)_3^{2+}$ to photoreduce Cu_A in cytochrome oxidase and examined the reduction of the P_M and F states (*24*). A wide range of ruthenium complexes have been developed to improve the yield of photoreduction of the oxidase. One approach is to increase the charge on the ruthenium complex to improve binding to the cytochrome *c* binding site on subunit II of the oxidase. The Ru_2C dimer complex with a charge of +4 was found to increase the yield of photoreduction of Cu_A by 5-fold compared to $Ru(bpy)_3$ (*22*). Another approach is to alter the bipyridine ligands in the ruthenium complex in order to tune the redox potentials of the complex and optimize the rate and yield of photoreduction. The Ru_2Z complex was designed to optimize both the electrostatic interaction with cytochrome oxidase and the driving force of the photoreduction reaction. The substitution of the bipyridine ligands with the bipyrazine ligands in Ru_2Z changed all four redox potentials of the complex by up to 0.5 V (Table 1). The redox potential of the II*/I transition was increased from 0.83 to 1.16 V, which allows the sacrificial electron donor aniline to reduce the excited state Ru(II*) to Ru(I). The Ru(I) can then directly reduce Cu_A according to scheme in Figure 3. The yield of photoreduction of the oxidase by Ru_2Z is over 70% under anaerobic conditions. Ru(I) is also reduced by oxygen, accounting for the decrease in the yield of photoreduction of the oxidase to 60% under aerobic conditions.

One-Electron Photoreduction of Cytochrome Oxidase. The problem being addressed with the new photoreductant is the nature of the "reductive" steps in the catalytic cycle. All current models of proton pumping ascribe one proton as being pumped for each of the four electrons entering the heme a_3/Cu_B center required to reduce O_2 to $2H_2O$. The net reaction is

$$O_2 + 8H^+_{in} + 4e^{-1} \rightarrow 2H_2O + 4H^+_{out}$$

where "in" refers to the bacterial cytoplasm (or mitochondrial matrix) and "out" refers to the bacterial periplasm (or mitochondrial intermembrane space). For each electron delivered to the active site (indicated by Fe/Cu), the reaction can be written as

$$Fe/Cu{-}X + 2H^+_{in} + 1e^{-1} \rightarrow Fe/Cu{-}X[H] + 1H^+_{out}$$

where X represents the electron/proton acceptor at the heme a_3/Cu_B active site, which is different for each step of the reaction. Starting with the fully oxidized enzyme, it is necessary to reduce the enzyme by two electrons in order to initiate the reaction with O_2. However, based on the equilibrium midpoint potentials of the redox centers, there is very little driving force (ΔG) to move electrons from reduced cytochrome *c* to the heme a_3/Cu_B center prior to the reaction with O_2 and certainly not enough to drive protons across the membrane against an electrochemical potential that may be as high as 220 mV (negative inside). The Wikström laboratory (*28*, *29*, *39*, *40*) has provided evidence that the reaction of the fully reduced enzyme (R_4) with O_2 generates a state of the oxidized enzyme that is transiently "activated", called the O_H or H state, such that the midpoint potential of Cu_B is significantly more positive than the equilibrium value. This increased midpoint potential is proposed to provide the free energy to pump a proton coupled to the $O_H \rightarrow E$ transition, during which an electron is transferred from cytochrome *c* to Cu_B. The explanation of the energetics of coupling proton pumping to the second electron, i.e., the $E \rightarrow R_2$ transition, is not clear, though evidence indicates that this step is also coupled to the pump (*30*). In the presence of O_2, the two-electron reduced oxidase reacts, giving a net reaction of E $(+e^{-1}) \rightarrow R_2$ $(+O_2) \rightarrow P_M$. Conceivably, the reaction to form P_M under these circumstances could provide sufficient free energy to pump a proton, though this seems not to be required (*30*).

The expectation is that the one-electron reduction of the oxidase in the O_H state will result in the electron residing on Cu_B due to the high midpoint potential of Cu_B in the activated state. While the current work was in progress, Belevich et al. (*28*) reported precisely this result, using the *P. denitrificans* oxidase. Photoinjection of one electron into the O_H form of *P. denitrificans* oxidase resulted in electron transfer from heme *a* to the heme a_3/Cu_B center, and this was coupled to proton translocation (*40*). However, no proton translocation is observed coupled to the one-electron injection into the nonpulsed O form of the enzyme (*39, 40*). Photoreduction of the O_H state resulted in rapid reduction of heme *a*, followed by biphasic electron transfer from heme *a* to the binuclear heme a_3/Cu_B center with rate constants of 6700 s^{-1} and 1250 s^{-1}, with equal amplitudes. Heme a/Cu_A was completely reoxidized. It was proposed that the first phase of electron transfer from heme *a* is coupled to proton translocation across the major part of the membrane, while the second phase was coupled to proton transfer from the inside of the membrane (electronegative side) to the heme a_3/Cu_B site, resulting in protonation of the OH^- ligand of Cu_B.

In the present studies, the electron-transfer kinetics were examined following one-electron injection into the O_H form of a number of different oxidase preparations. One-electron reduction of the O_H form of the bovine oxidase resulted in electron transfer from Cu_A to heme *a* with a rate constant of 20 000 s^{-1}, followed by biphasic electron transfer from heme *a* to the binuclear heme a_3/Cu_B center with rate constants of 750 s^{-1} and 110 s^{-1} and relative amplitudes of 25% and 75%. The total oxidation of heme a/Cu_A was 63%. The absence of any change in the 436 nm absorbance, an isobestic for heme *a*, indicates that the electron was not transferred to heme a_3 but rather to Cu_B. In contrast, one-electron injection into the nonpulsed fast bovine oxidase resulted in slower, monophasic reoxidation of heme *a* (90 s^{-1}), and the reoxidation of heme a/Cu_A was only 30% complete. The data demonstrate a significant difference between the pulsed O_H and nonpulsed O state of the fast bovine oxidase.

The present data stand in contrast to previous studies in which no differences were found in the properties of the O and O_H states of the fast bovine oxidase (*38*). In the previous studies, heme *a* and Cu_A are rapidly reduced within a few milliseconds by excess ruthenium hexamine, and electron transfer to the heme a_3/Cu_B center proceeds with a rate constant of about 80 s^{-1}, regardless of whether the enzyme is pulsed or not. Furthermore, rapid-quench EPR studies showed that the distribution of electrons between heme a_3 and Cu_B during the time required for full reduction of the heme a_3/Cu_B center (1 s) was not different for the pulsed and nonpulsed enzymes. This is also supported by observation of the 650 nm charge-transfer band, used to monitor the reduction of Cu_B. There is no rapid electron transfer to Cu_B, followed by slower reduction of heme a_3, since this would result in the rapid appearance of a large EPR signal due to high spin ferric heme a_3, and this is not observed. The main discrepancy between the present results and those of reference 38 is the presence of the small fast phase of reoxidation of heme *a* with a rate constant of 750 s^{-1} and an amplitude of 25%. The reason for this discrepancy is not clear, but it may be due to the injection of a single electron into oxidase by the flash method compared to the injection of multiple electrons by excess ruthenium hexamine. It is also possible that the fast phase is associated with a small fraction of oxidase in which heme a_3 remained reduced 20 ms after mixing with oxygenated buffer.

The rates of electron transfer from heme *a* to the binuclear center in the O_H form of the bovine enzyme observed in the current work (750 s^{-1} and 110 s^{-1}) are not nearly as fast as those observed with the *P. denitrificans* oxidase (6700 s^{-1} and 1250 s^{-1}) (*28*). Whether the observations in the current work represent the equivalent phenomenon reported (*28*) for the *P. denitrificans* oxidase cannot be ascertained at this time. If the effects observed in the current work represent a transient activation of the enzyme, it can be concluded that the properties of the O_H state must persist for at least 100 ms.

The same experiments carried out with the bacterial oxidases from *R. sphaeroides* and *P. denitrificans* did not show any significant differences between the O_H and O forms of the enzymes. One-electron injection into the O_H form of the *R. sphaeroides* oxidase led to monophasic electron transfer from heme *a* to the binuclear center with a rate constant of 760 s^{-1} and an extent of reoxidation of heme *a* of only 26%. Nearly the same results were obtained with the nonpulsed O form of the enzyme, with a rate constant of 1000 s^{-1}. Enrichment of the *R. sphaeroides* oxidase preparation with cardiolipin and phosphatidyl choline led to an increase in the rate constant of electron transfer from heme *a* to the heme a_3/Cu_B center but no significant difference between the pulsed and nonpulsed states.

Since the work of Belevich et al. (*28*) demonstrating a dramatic difference between the O_H and O states of the enzyme was performed with the *P. denitrificans* oxidase, the current work was extended to include this enzyme as well (kindly provided by B. Ludwig, University of Frankfurt). The extent of reoxidation of heme a/Cu_A was also relatively small in a preparation of the *P. denitrificans* oxidase in the O_H form, with a rate constant of 3000 s^{-1} and a reoxidation extent of only 25%. Hence, the differences in the current results and those of Belevich et al. (*28*) do not reside in the origin of the enzyme.

The reason for the difference between the present results and those of Belevich et al. (*28*) is not known. However, Belevich et al. do state that their results were highly dependent on the individual preparations used in the experiments and gave variable results. Some preparations of the *P. denitrificans* oxidase reported in reference 28 demonstrated behavior similar to those reported in the current work, and consistent results were obtained only with solubilized membranes, avoiding the rigors of column chromatography of the enzyme. Efforts to perform similar experiments with *R. sphaeroides* membranes were not successful, likely due to the low concentration of the oxidase in these membranes. It seems, however, that the membrane environment of the oxidase is critical to demonstrating the properties of the O_H state in the bacterial oxidases and perhaps also for the bovine oxidase. However, Jancura et al. (*38*) observed no difference in the spectral and kinetic properties of the O and O_H states of bovine oxidase in the natural membrane environment.

Interestingly, although the rapid and complete reoxidation of heme *a* of the bacterial oxidases was not observed in the O_H state, the enzyme has been shown many times to pump protons with a stoichiometry close to $1H^+/e^{-1}$ during steady-state turnover. Hence, if this requires the "activated" O_H state of the enzyme, then the *R. sphaeroides* oxidase must pass through this state transiently during steady-state turnover. The failure to demonstrate the expected properties in the one-electron photoreduction experiment apparently does not correlate with whether the same enzyme preparation exhibits full proton pumping during steady state.

Two-Electron Reduction of Cytochrome Oxidase. There is no evidence from this work or from previous studies that the one-electron reduced oxidase reacts with O_2, presumably because the electron does not reside on heme a_3. It is well-documented that the two-electron reduced enzyme does react with O_2, forming the P_M intermediate. The two-electron reduced oxidase is most often prepared by using CO as a reductant and forming the CO adduct of the "mixed valence" state of the enzyme (*51*). In this state the two electrons reside on heme a_3 and Cu_B. CO is ligated to the ferrous heme a_3 iron. Photolysis to remove the CO in the presence of O_2 results in rapid reaction of this two-electron reduced form of the enzyme to yield the P_M state (*52*). However, this reaction is not electrogenic and does not pump protons (*53*).

In the current work, a protocol is demonstrated to generate a two-electron reduced form of the oxidase without using CO but by photoreduction of the one-electron reduced state. This procedure should mimic the steps during steady-state turnover. A mutant form of horse heart cytochrome *c* (K13E) is used under conditions which stoichiometrically reduces the enzyme but which dissociates rapidly, allowing Ru_2Z to bind to the same site on the oxidase. It is expected that some of the oxidase might be reduced by two electrons by the K13E cytochrome *c*, but this appears not to be the case. By preforming the 1:1 complex between cytochrome *c* and the oxidase prior to oxidation by O_2, a high yield of the one-electron reduced (E) form of the enzyme is formed. Photoreduction of Ru_2Z results in a high yield (about 40%) of the two-electron reduced state (R_2), which reacts rapidly with O_2 to form the P_M state of the enzyme. This appears to proceed to completion, judging from the transients at 605 and 436 nm. Using this protocol to inject two electrons into the oxidase through Cu_A, it is expected that this reaction should be electrogenic and pump protons, in contrast to the reaction of O_2 with the CO-mixed valence enzyme which starts with a prereduced heme a_3/Cu_B center. Future studies will examine this reaction using the enzyme reconstituted in phospholipid vesicles to directly measure proton pumping coupled to the steps in the catalytic cycle prior to the formation of the P_M intermediate.

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