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Short-lived neutral FMN and FAD semiquinones are transient intermediates in cryo-reduced yeast NADPH-cytochrome P450 reductase

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Abstract

The electron configuration of flavin cofactors, FMN and FAD, is a critical factor governing the reactivity of NADPH-cytochrome P450 reductase (CPR). The current view of electron transfer by the mammalian CPR, based on equilibrium redox potentials of the flavin cofactors, is that the two electron-reduced FMN hydroquinone (FMNH2), rather than one electron-reduced FMN semiquinone, serves as electron donor to the terminal protein acceptors. However, kinetic and thermodynamic studies on the CPR species originated from different organisms have shown that redox potentials measured at distinct electron transfer steps differ from redox potentials determined by equilibrium titration. Collectively, previous observations suggest that the short-lived transient semiquinone species may carry electrons in diflavin reductases. In this work, we have investigated spectroscopic properties of the CPR-bound FAD and FMN reduced at 77 K by radiolytically-generated thermalized electrons. Using UV-vis spectroscopy, we demonstrated that upon cryo-reduction of oxidized yeast CPR (yCPR) containing an equimolar ratio of both FAD and FMN, or FAD alone, neutral semiquinones were trapped at 77 K. During annealing at the elevated temperatures, unstable short-lived neutral semiquinones relaxed to spectroscopically distinct air-stable neutral semiquinones. This transition was independent of pH within the 6.0 – 10.7 range. Our data on yeast CPR are in line with the previous observations that the flavin short-lived transient semiquinone intermediates may have a role in the electron transfer by CPR at physiological conditions.

Keywords

CPR; cryo-reduction; absorption spectroscopy; neutral flavin semiquinones; short-lived neutral flavin semiquinones

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NADPH-dependent cytochrome P450 reductase (CPR) belongs to the diflavin reductase protein family. It has an important physiological role as a key electron transporter to the cytochrome P450 monooxygenases (CYPs) in the endoplasmic reticulum that play a central role in metabolism of endogenous chemicals and xenobiotics including drugs. CYPs catalyze the insertion of one atom of molecular oxygen into their substrates with the reduction of the other atom to water, a reaction requiring two electrons that are delivered by CPR. Other physiological electron acceptors of CPR are cytochrome b5, NOS oxygenase domain, and heme oxygenase.[1–4] CPR contains both FMN and FAD cofactors and uses NADPH as a source of electrons. CPR consists of three protein domains: an FMN-binding domain, an FAD and NADPH-binding domain, and a “connecting” domain. The FMN domain is linked to the connecting and FAD domains through a flexible “hinge.”[1, 3, 4] The FMN domain of CPR is homologous to flavodoxin, while the FAD domain is homologous to ferredoxin-NADPH reductase.[1] CPR transfers two reducing equivalents, in the form of a hydride ion derived from NADPH, to FAD, and the latter transfers electrons to FMN, from where they are delivered to acceptor proteins in two one-electron transfer steps.[2]

One FMN binding site and one FAD binding site were identified in mammalian CPRs.[1, 5] The yeast CPR (yCPR) x-ray structure has revealed, in the presence of externally added FMN, a second FMN binding site (FMN2-site), having different accessibilities and amino acid environments, the latter suggesting potential to stabilize different electronic forms of the reduced FMN.[6] The canonical FMN binding site (FMN1-site) is similar to that in mammalian CYPs and flavodoxins, whereas the FMN2-site is located in the interface between the FMN binding and connecting CPR domains and may be accessible from the protein surface.[6, 7]

The three-ring flavin moiety, isoalloxazine, can exist in three different oxidation states: oxidized (ox), one-electron reduced semiquinone (sq) and 2-electron reduced hydroquinone (hq). The flavin semiquinones can be in an anionic (red) or in neutral (blue) state in which the N5 position of isoalloxazine is protonated. The protonation at N5 causes significant changes in UV-vis absorbance and EPR spectra.[8–10] The EPR studies of anionic and neutral semiquinones indicate that protonation of N5 affects spin distribution of an unpaired electron in isoalloxazine ring.[9, 11, 12]

An anionic semiquinone is characterized by absorption maxima at ~360 nm and ~480 nm, while the neutral semiquinone may exist in two spectrally distinct forms: inert air-stable neutral semiquinone and highly reactive short-lived neutral semiquinone. The air-stable neutral semiquinone shows a broad maximum at ~590 nm ascribed to a π- π^* transition with vibronic features around 600–630 nm.[8–10] The short-lived neutral semiquinone shows broad maxima around 570 nm and 610 nm.[8, 10] In CPR, the air-stable neutral semiquinone forms of FAD and FMN can be spectrally distinguished by the presence of a 630 nm vibronic component, which is poorly resolved in the FAD semiquinone.[1, 11, 12]

The local protein environment significantly influences the FMN redox potential, accounting for differences between flavin enzymes.[2, 5, 11–16] In mammalian CPRs[5, 11, 12] and flavodoxins[13], the Eox/sq midpoint potential shifts from −313 mV (in free FMN) to –(60–

100) mV, while the Esq/hq potential changes from −100 mV to −252 mV[5]. Modulation of the FMN redox potential by the protein environment may explain the air stability of neutral FMNsq (FMNH) and the high activity of FMNhq ($FMNH_2$) in the reduction of P450 by human CPR.[5, 11, 12]

Crystal structures of wild-type and site-directed mutants of flavodoxins from *Clostridium beijerinckii* showed that the conformation of the peptide Gly57-Asp58 near the isoalloxazine ring of FMN changes with the oxidation of the FMN cofactor.[13, 14, 16] In oxidized flavodoxin, Gly57-Asp58 adopts a conformation in which the carbonyl oxygen of Gly57 points away from N5. In the neutral semiquinoid form and the hydroquinoid forms, both of which have N5 protonated, the Gly57 carbonyl oxygen forms an H-bond to the flavin N(5)H. Similar observations were made in studies of recombinant human CPR lacking Gly141 in the conserved active site loop formed by peptide G141-G143 bound nearby to the FMN.[5, 17] These observations support the hypothesis that hydrogen bonding between the carbonyl of Gly141 and N(5)H of neutral FMN radical stabilizes the air-stable form. Another factor affecting the redox properties of flavin cofactors is aromatic stacking interactions with the isoalloxazine ring.[18–20]

All one-electron-reduced CPRs can in equilibrium stabilize an air-stable neutral semiquinone that does not efficiently reduce P450 or cytochrome *c*.[2] However, kinetic and thermodynamic studies have shown that redox potential at distinct electron transfer steps may differ from the redox potential determined in equilibrium.[2] This observation opens a possibility that the short-lived semiquinoid FMN intermediates with lower redox potential and higher reactivity may be involved in electron transfer to terminal electron acceptors.

To trap short-lived semiquinoid intermediates, we reduced the flavin-containing recombinant yCPR at 77 K by radiolytically-generated thermalized electrons and studied spectroscopic properties of the yCPR-bound cofactors upon annealing at higher temperatures. We demonstrated that 77 K cryo-reduction of the oxidized flavin cofactors bound to yCPR (yCPRox) resulted in formation of unstable neutral flavin semiquinone(s) trapped in a metastable protein environment pertinent to oxidized CPR. During annealing at temperatures ranging from 77 K to 270 K, the protein environment relaxed, leading to formation of a spectroscopically distinct air-stable neutral flavin semiquinone. Our results are in line with the observations of the short-lived transient flavin semiquinone intermediates in housefly CPR and cytochrome P450 BM3, were their potential physiological role in electron transfer by diflavin reductases was suggested.[2, 15]

Materials and methods

FMN, FAD, sodium dithionite, potassium and sodium phosphates, glycerol and other reagents of common use were purchased from Sigma and used without further purification.

Yeast CPR purification

The truncated form of yCPR lacking 33 amino acids at the N-terminus and containing six N-terminal histidine residues was expressed in *E. coli* and was purified to homogeneity by affinity chromatography on Ni-NTA agarose (Qiagen), followed by ion-exchange

chromatography on S-Sepharose and Q-Sepharose, as previously described.[6] The purified holo-yCPR used in this work contained tightly bound cofactors FMN/FAD in equimolar ratio.[6] The FMN-depleted yCPR was prepared by extensive dialysis of the purified recombinant yCPR at +4 °C against 2 M KBr in 50 mM pyrophosphate buffer, pH 8.5, containing 5% glycerol, as described elsewhere.[12] No chaotropic agents were used. The FMN depletion was monitored by the drop in absorbance at 473 nm (ε=9200 $M^{-1}cm^{-1}$ for both FMN and FAD[12]). A plateau at ~60% of the starting absorbance value was reached, suggesting that ~80% of FMN was dialyzed out. The dialyzed sample was transferred to 50 mM potassium phosphate buffer, pH 7.5, and an additional purification step using Q-Sepharose was performed. Protein eluted from Q-Sepharose was concentrated to ~2 mM, frozen at −80 °C and stored until needed.

Cryogenic spectroscopy

Cryogenic radiolytic reduction.—For cryogenic reduction, all samples were prepared in a buffer/glycerol (1:1.5 v/v) mixture forming a transparent glass at 77 K. Cylindrical quartz tubes with OD 4.33 mm or 2.10 mm were used for sample freezing. The final concentration of yCPR or free flavin samples was 0.4 mM. The frozen samples kept in a Dewar flask filled with liquid nitrogen were irradiated by γ-rays from a Gammacell 220 ^{60}Co source (Notre Dame Radiation Laboratory) (dose rate of 0.5 Mrad/hr) for 6 hr. A total dose of 3 Mrad was typically achieved.

Annealing.—Annealing at temperatures over the range of 77–270 K was performed by placing the irradiated sample in the appropriate bath (e.g., *n*-pentane or methanol cooled with liquid nitrogen) followed by re-freezing in liquid nitrogen. The trapped electrons formed during γ-irradiation, giving strong absorbance in the visible range, were photo bleached prior to spectroscopic measurements by illuminating the sample with the white light of a 100 W halogen lamp.

Optical spectra at 77 K were recorded in a cold finger quartz Dewar, filled with liquid nitrogen, using an Ocean Optics USB 2000 spectrophotometer. The X-band CW electron spin resonance spectra were recorded on Bruker ESR-300 spectrometer equipped with an Oxford Instruments ESR 910 continuous He flow cryostat.

Cryogenic radiolytic oxidation.—γ-Irradiation at 77 K of proteins carrying redox centers yields both one-electron-reduced and one-electron-oxidized products.[21] Radiolytic cryo-oxidation was performed by γ-irradiation at 77 K of fully reduced yCPR prepared by reduction of oxidized protein with 2-fold molar excess of dithionite.[21] The yield of the cryo-oxidized yCPR was low compared to cryo-reduced yCPR due to (i) mechanistic differences between the cryo-oxidation and cryo-reduction processes[21–23] and (ii) subsequent reduction of the cryo-generated oxidized species by radiolytically-generated electrons.

Results

UV-vis spectroscopy of free FMN and FAD reduced at 77 K.

The low temperature UV-vis absorbance spectra of oxidized FMN (FMNox) and FMN exposed to γ-irradiation at 77 K are shown in Fig. 1. FMNox shows absorbance maxima at 379 nm and 444 nm and a well-resolved shoulder at 465 nm (red trace). γ-Irradiation of FMNox at 77 K with a dose of 3 Mrad resulted in a decrease of intensities of the FMN bands by a factor of ~1.7 and gave rise to an absorbance with broad bands at 563 nm and 593 nm, characteristic of a short-lived neutral flavin semiquinone (Fig.1, black trace).[8, 10] The low temperature spectrum of the radiolytically-generated FMN semiquinone (FMNsq) trapped at 77 K was obtained by subtracting a scaled FMNox spectrum from the spectrum of γ-irradiated FMN (Fig. S1). The calculated spectrum was similar to a spectrum of FMNsq at ambient temperature reported elsewhere.[8, 10]

At pH 11.5, the band intensity in the cryo-generated neutral FMNsq spectrum notably decreased (Fig. S2) Annealing of the frozen cryo-generated FMNsq at temperatures between 145 K and 195 K for 0.5–2 min had no effect on the optical absorbance spectra (Fig. S3). Upon further annealing at T > 200 K, the water/glycerol glass lost transparency, impeding spectroscopic measurements. After further annealing at 293 K for 5 min, the sample had liquefied and became transparent again, revealing an absorbance spectrum characteristic of FMNox (Fig. S3). Disappearance of FMNsq was likely due to disproportionation. Similar data were obtained for cryo-reduced FAD (Table.1).

UV-vis spectroscopy of yCPR reduced at 77 K.

The 77 K UV-vis spectra of yCPRox and yCPRox exposed to γ-irradiation at 77 K are presented in Fig. 2. The yCPRox spectrum shows absorbance maxima at 384 nm and 456 nm and a well-resolved shoulder at 478 nM (red trace). γ-Irradiation of yCPRox at 77 K resulted in decreased intensity of the spectrum by a factor of ~2 and gave rise to a new absorbance maximum at 570 nm and a shoulder at 608 nm (cyan trace), characteristic of a short-lived neutral flavin semiquinone.[8–12, 24–27] A spectrum of the cryo-radiolytically generated one-electron reduced CPR trapped at 77 K was obtained by subtracting a scaled yCPRox spectrum from the raw spectrum of the γ-irradiated yCPR (Fig.S4). This operation allowed better resolution of the 570 nm and 607 nm peaks and revealed an additional weak peak at 506 nm (Fig.S4)). The nature of the latter remains unclear: it may be an artifact of the mathematical operation or a result of the formation of small amounts of anionic flavin semiquinone.

The absorbance above 500 nm in the optical spectra of the cryo-reduced yCPR is essentially the same as that of the cryo-generated free flavin semiquinone, with the peak maxima slightly red-shifted. (Figs. 1 and 2, Table 1). The absorbance spectra of cryo-reduced yCPR were independent of pH within the pH range where yCPRox is stable (pH 6.2–10.7) (Fig.S5). Because the radiolytically-generated electrons interact randomly with both flavin cofactors in yCPR, equal amounts of FMN and FAD semiquinones are expected to accumulate in cryo-reduced yCPR. Thus, the optical spectrum of cryo-reduced yCPR in Fig.2 is likely a sum of the neutral FMNsq and neutral FADsq spectra.

Annealing of cryo-reduced yCPR at elevated temperatures.

Stepwise annealing between 145 K and 175 K, each for 2 min, had no effect on the optical absorbance spectra of the flavin semiquinones of cryo-reduced yCPR (green and pink traces in Fig. 3). Upon further annealing at ~190 K for ~45 s (as long as the water/glycerol glass remained transparent), the flavin semiquinones absorbance maxima shifted to 575 nm and 623 nm, respectively (Fig. 3, blue trace). After annealing at 293 K for 5 min, cryo-reduced yCPR showed an absorbance spectrum with two well-resolved maxima at 581 nm and at 628 nm characteristic of an air-stable semiquinone (Fig. 3, golden-yellow trace) with ~2-fold increase in intensities compared to the cryo-reduced yCPR prior to annealing. The latter may be explained by the electron transfer from cryo-generated FADsq to oxidized FMN. The presence of molecular oxygen had no effect on the 293 K-annealed spectrum. The 293 K annealed spectrum resembled spectra reported for the air-stable semiquinone generated elsewhere by reduction of mammalian CPRs at ambient temperatures, photochemically or with NADPH or sodium dithionite (Table 1).[1, 5, 11, 12, 24, 26]

From previous experience, a cryo-reduction/annealing cycle has a minor effect on the catalytic activity of heme-containing enzymes.[32–35] Similarly, yCPR subjected to radiolytic cryo-reduction/annealing cycle retained ~90% activity in the reaction of benzphetamine oxidation catalyzed by rat CYP2B4.[5,17]

UV-vis spectroscopy of FMN-depleted yCPR reduced at 77 K.

To evaluate the contribution of FAD to the absorbance spectra of yCPR, we conducted experiments with FMN-depleted yCPR. Similar to the wild-type containing both FMN and FAD, the absorbance spectrum of the cryo-reduced FMN-depleted yCPR showed long wavelength maxima at 573 and 610 nm, characteristic of the neutral semiquinone, with the qualification that the shoulder at 610 nm was less resolved (compare Fig. 4 with 1 and 2). The cryo-reduced FMN-depleted yCPR had an annealing pattern similar to that of the wild-type yCPR (compare Fig. S6 A and Fig. 3).

An absence of electron transfer from FAD to FMN allowed us to resolve an isosbestic point at 550 nm during annealing of the cryo-reduced FMN-depleted yCPR at temperatures above 185 K (Fig. 6B). The presence of the isosbestic point indicates that the cryo-generated neutral FAD semiquinone converted directly into the air-stable form during sample annealing.

Protonation status of flavin semiquinones assessed by EPR spectroscopy.

Formation of matrix radicals during the 77 K γ-irradiation of free FMN and FAD precludes application of EPR spectroscopy to study the cryo-generated free flavin semiquinones. Therefore, we measured EPR spectra of the FMNsq formed in ethanol upon sample illumination at 20 K in the EPR cavity using a 20 mW photodiode with a wavelength of 450 nm. After 20 min of illumination, the sample showed an EPR spectrum with a peak-to-peak line width of 21 G (Fig. S7) characteristic of neutral flavin semiquinone with protonated N5. [28, 29]

Similarly, the EPR spectrum of yCPR cryo-reduced at 77 K was obscured by signals from the irradiated matrix. However, raising the temperature to 293 K destroyed the matrix radicals, and allowed EPR examination of the yCPR-bound flavin semiquinones. The cryo-generated air-stable yCPR-bound FMNsq in H_2O solution showed an EPR signal with a peak-to-peak line width of 21 G (Fig. S6), implying a neutral flavin semiquinone.[28, 29] Air-stable semiquinone was confirmed by a decrease in the linewidth of the signal to ~15.5 G in D_2O solvent caused by the proton-deuterium exchange at N5. The EPR signal of the annealed cryo-reduced FMN-depleted yCPR was very close to that of the free FMN cofactor (Fig. S7).

UV-vis spectroscopy of cryo-reduced glucose oxidase.

Glucose oxidase was used in this work as a comparative companion for yCPR. Unlike yCPR, glucose oxidase contains only an FAD cofactor that forms relatively stable red and blue semiquinones upon photochemical reduction under anaerobic conditions at pH 10.35 and 6.65, respectively.[9, 10] The same experiments described above for yCPR were performed with glucose oxidase. When glucose oxidase containing an oxidized cofactor was cryo-reduced at pH 7.5, it only showed weak absorbance at wavelengths above 550 nm corresponding to a neutral FAD semiquinone (Fig.5). However, the cryo-reduced protein revealed well-resolved absorbance peaks at ~ 370 nm characteristic of anionic semiquinone (Fig. 5). After annealing at T > 180 K, the intensity of the red semiquinone peak decreased and new peaks at 565 and 605 nm corresponding to a short-lived neutral semiquinone appeared in the absorbance spectrum. Addition of molecular oxygen resulted in complete disappearance of the semiquinone signal.

Cryo-oxidation of fully-reduced yCPR.

The spectral differences between cryo-generated FMN and FAD semiquinones prior to and after annealing likely reflect changes in the interactions of the isoalloxazine ring of the cofactors with the protein environment. To test this hypothesis, we cryo-oxidized fully-reduced yCPR by γ-irradiation at 77 K. The sodium dithionite-reduced yCPR showed an absorbance spectrum characteristic of a two-electron reduced hydroquinone with the peak at 428 nm (Fig. 6, red trace). γ-Irradiation resulted in appearance of absorbance maxima at 580 and 630 nm (Fig. 6, blue trace), characteristic of air-stable neutral semiquinone(s). The air-stable FMN semiquinone formed during cryo-oxidation of fully-reduced yCPR did not significantly change during annealing.

Discussion

Radiolytic cryo-reduction of yCPR allowed us to generate, trap, and spectroscopically characterize semiquinoid flavin intermediates that play key roles in the functions of various flavoproteins. At 77 K, the dominant form of the cryo-reduced FMN and FAD, both free and yCPR-bound, were short-lived neutral semiquinones, as judged by the characteristic band at 570 nm and a shoulder at 607 nm in the UV-vis absorbance spectra (Fig. 1–4, Table 1). Similar to the wild-type, the absorbance spectrum of the cryo-reduced FMN-depleted yCPR showed long wavelength maxima at 573 and 610 nm, characteristic of the FAD neutral semiquinone. Similar spectra were reported for the neutral flavin semiquinones trapped

during the x-ray data collection at 100 K for rat CPR[30] and for the *Bacillus cereus* flavoenzyme, NrdI[31]. The absorbance spectra of cryo-reduced yCPR were independent of pH within the range of 6.2–10.7 (Fig.S5). An increase in pH would favor formation of anionic semiquinone. In glucose oxidase, basic pH did favor formation of the anionic semiquinone characterized by a distinct peak at ~370 nm (Fig. 5).

Trapping transient neutral semiquinones during radiolytic cryo-reduction of yCPR may be explained either by reduction of the oxidized flavins already protonated at N5, or by proton transfer to the cryo-generated anionic semiquinone. Protonation of the oxidized flavin at N5 is characterized by pKa < 0.25.[8, 30, 31] } It seems unlikely that low temperature or proximity of protein functional groups would shift the pKa of N5 by 8–10 units. A more realistic scenario is that proton transfer in yCPR occurred to cryo-reduced flavins. A neutral flavin semiquinone radical is also a primary species formed during the rapid reduction of mammalian CPR by a photo-generated 5-deaza riboflavin radical at pH 7.0.[24] Further, proton transfer to a peroxy ferriheme species generated during radiolytic cryo-reduction at 77 K of oxy complexes of ferrous peroxidase and heme monooxygenases, is often observed. [32–34]

The peak maxima in the absorbance spectra of the cryo-generated yCPR-bound FMN and FAD semiquinones are red-shifted by ~10 nm compared to the corresponding free flavins. [20–23, 30, 31, 33, 35, 36] This spectral shift may be explained by the influence of the protein environment.[37] Additional spectral changes were observed upon annealing cryo-reduced yCPR at temperatures above 180 K (Fig. 4, S5, Table 1). The annealed cryo-reduced yCPR showed absorbance spectra characteristic of an air-stable semiquinone reported previously for mammalian CPRs and flavodoxins. This spectrum features a well-resolved long wavelength $\pi - \pi^*$ transition band at ~590 nm and a vibronic band at ~630 nm (Table 1, Fig. 3, 4).[1, 5, 11, 12, 26, 38] At the same time, the neutral semiquinone of annealed cryo-reduced glucose oxidase exhibited absorbance peaks very similar to those in cryo-reduced free flavins. This once again demonstrated that formation of an air-stable semiquinone depends on protein environment. We speculate that cryo-reduction of oxidized yCPR produces semiquinones trapped in the protein conformation associated with the oxidized quinoid form. The spectroscopic changes observed during annealing cryo-reduced yCPR reflect relaxation of the protein environment to a conformation in equilibrium with the semiquinone.

The structural determinants of the flavin semiquinone optical spectra are not fully established. They may include isoalloxazine ring bending, aromatic stacking with amino acid side chains, polarity, hydrogen bonding networks and electrostatic interactions provided by the nearby amino acid residues.[20, 37, 39] The isoalloxazine ring bending is not likely to be responsible for the spectral changes observed during relaxation of the cryo-reduced flavin cofactors. The absorbance maxima for neutral flavin semiquinones in the visible range (above 500 nm) depends on the flavoprotein and varies from 570 nm in glucose oxidase,[40] to 610 nm in the *Bacillus cereus* flavoenzyme, NrdI,[31] and to 630 nm and 675 nm in *C. reinharddii* LOV1.[40] Comparison of the flavodoxin structures in different oxidation states indicates that FMN remains planar in all three oxidations states.[16] This conclusion is supported by the spectral similarities of semiquinone radicals generated photochemically.

[41] It is further supported by the observation that the absorbance spectrum of the flavin semiquinone formed during radiolytic cryo-oxidation of reduced yCPR is similar to that of the air-stable FMNH cofactor (Fig.6).

The hydrogen bonding interaction between the semiquinone N(5)H and the carbonyl of an amino acid residue from a conserved flexible polypeptide loop in its vicinity is an important structural factor modifying the redox potential and pKa of CPR-bound neutral FMN semiquinone.[5, 16] Site-directed mutations of the loop amino acid residues, which disrupt such H-bonding, were shown to alter the redox potential and pKa of the CPR-bound neutral FMNH cofactor, but had little effect on its absorbance spectrum.[5, 16, 19, 20] According to the crystal structures of flavoproteins, the quinoid and semiquinoid forms of the flavin cofactor differ by an H-bond between the protein carbonyl group and N5(H) of the flavin cofactor.[16] However, the formation of such an H-bond in the semiquinone form is not likely the cause of the spectroscopic shifts upon annealing.

Thus, it is likely that changes in the polarity of the binding site, as modulated by π-interactions between flavin semiquinone and stacking aromatic residues, make the main contribution to the changes of the absorbance spectrum of the cryo-generated FMN radical upon relaxation of the active site during annealing. FMN and FAD cofactors in flavodoxin and CPR are ‘sandwiched’ between the two aromatic residues, tyrosine and tryptophan. Similarly, *Anabaena* and *D. vulgaris* flavodoxins[19, 20] have aromatic residues that contact the FMN cofactor. Site-directed mutagenesis of these aromatics has shown that they stabilize the neutral FMN semiquinone. In addition, tyrosine replacement for alanine, leucine or phenylalanine influenced the shape and position of maxima in the visible region of the protonated FMN semiquinone.[18–20] This suggests that the changes of absorbance spectra of the cryo-generated FMN radical upon annealing may also be caused by changes of polarity of the binding site modulated by π-interactions between flavin semiquinone and stacking aromatic residues.

This hypothesis is supported by model data on the influence of solvent polarity on the absorbance spectra of the N(5)alkyl-lumiflavin semiquinone.[40] Replacement of water for ethanol or benzene results in dramatic changes in shape and long-wave band positions.[40] In water, N(5)alkyl-lumiflavin neutral semiquinone shows maxima at 502 nm and 580 nm and a vibronic shoulder at ~620 nm. In less polar ethanol and benzene, two well-resolved peaks appear at 600 and 630 nm, and at 610 and 655 nm, respectively. Thus, a decrease in the polarity of the environment is consistent with the transformations observed in the spectra of cryo-reduced yCPR during the course of annealing. The similarity of spectral changes observed during annealing of both the wild-type and FMN-depleted cryo-reduced yCPR suggests similarity of factors affecting spectral changes of both semiquinones based on aromatic stacking interactions.

Cryo-reduction at 77 K generates a neutral semiquinone, equivalent to the product of a proton-coupled electron transfer (PCET), and it corresponds to the process that can occur in ambient temperature reduction. The measurements here show that the initial product of this PCET resides in a non-equilibrium protein environment, which then relaxes to the equilibrium semiquinone state. Is such a non-equilibrium state of functional importance?

The functional role of a short-lived flavin semiquinone in electron transport is determined by its lifetime and reactivity. The cryo-generated short-lived FMN and FAD cofactor semiquinones relaxed to an equilibrium state after annealing at ~ 200 K for ~ 1 min. Non-equilibrium states with similar relaxation properties were previously reported to form during cryo-reduction of several iron containing metalloproteins (cytochrome *c*, ferredoxin, myoglobin, hemoglobin).[40, 42, 43] Pulse radiolysis studies showed that such non-equilibrium states of these metalloproteins also form at ambient temperatures, and then relax to equilibrium states on a millisecond time scale.[40, 42, 43] In most cases, reactivity of the reduced metalloproteins toward different oxidants was 1–2 orders of magnitude higher in non-equilibrium states than at equilibrium.[40, 42, 43] By analogy with the non-equilibrium forms of metalloproteins, we suggest the non-equilibrium intermediate forms of the semiquinoid yCPR may have a similar life-time, lower redox potential and higher reactivity than the equilibrium air-stable neutral semiquinone.

Conclusions

The current view of electron transfer by the mammalian CPR based on equilibrium redox potentials of the flavin cofactors, is that the hydroquinone, $FMNH_2$, rather than semiquinones, serves as an electron donor to the terminal acceptor.[2] However, kinetic and thermodynamic studies on mammalian CPR have shown that redox potential changes during catalytic steps differ from those determined by redox equilibrium titration.[2] Kinetic studies on cytochrome *c* reduction by housefly CPR demonstrated that an unstable neutral semiquinone is an active intermediate while air-stable semiquinone reduces cytochrome c much more slowly.[44] Similar to cytochrome P450 BM3 and housefly CPR[2, 15, 44], our data suggest that the non-equilibrium semiquinoid form of yCPR, that first arises upon reduction of the quinoid form, can serve as the active species along with hydroquinone in the reduction of cytochrome P450. A functional role of the short-lived semiquinone is also supported by single molecule activity measurements of CPR from *Sorghum bicolor* reconstituted in lipid nanodiscs with resazurin as electron acceptor. Those studies revealed the existence of two discrete functional states masked in bulk kinetics because of ensemble averaging.[45] The activity of these two states differs by a factor of ~20. It was found that CPR spends 5% of its time in the highly active reduced state.[45] Such intermediate states were also detected during photo-induced electron transfer from stacking aromatic residues to a neutral FMN semiquinone cofactor in flavodoxin. In this process, electron transfer is coupled with local environment relaxation, and is followed by stretched-exponential behavior.[18] The results presented here, in conjunction with the given examples, suggest that non-equilibrium short-lived states of the flavin semiquinones may also be involved in electron transfer carried out by diflavin reductases at physiological conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Short-lived neutral FAD and FMN semiquinones were trapped upon cryo-reduction of yeast CPR at 77 K
- Upon annealing, unstable neutral semiquinones relaxed to air-stable neutral semiquinones
- The absorbance spectra of cryo-reduced yeast CPR were independent of pH within the 6.0–10.7 range
- Transient flavin semiquinone intermediates may serve as reactive species in CPR

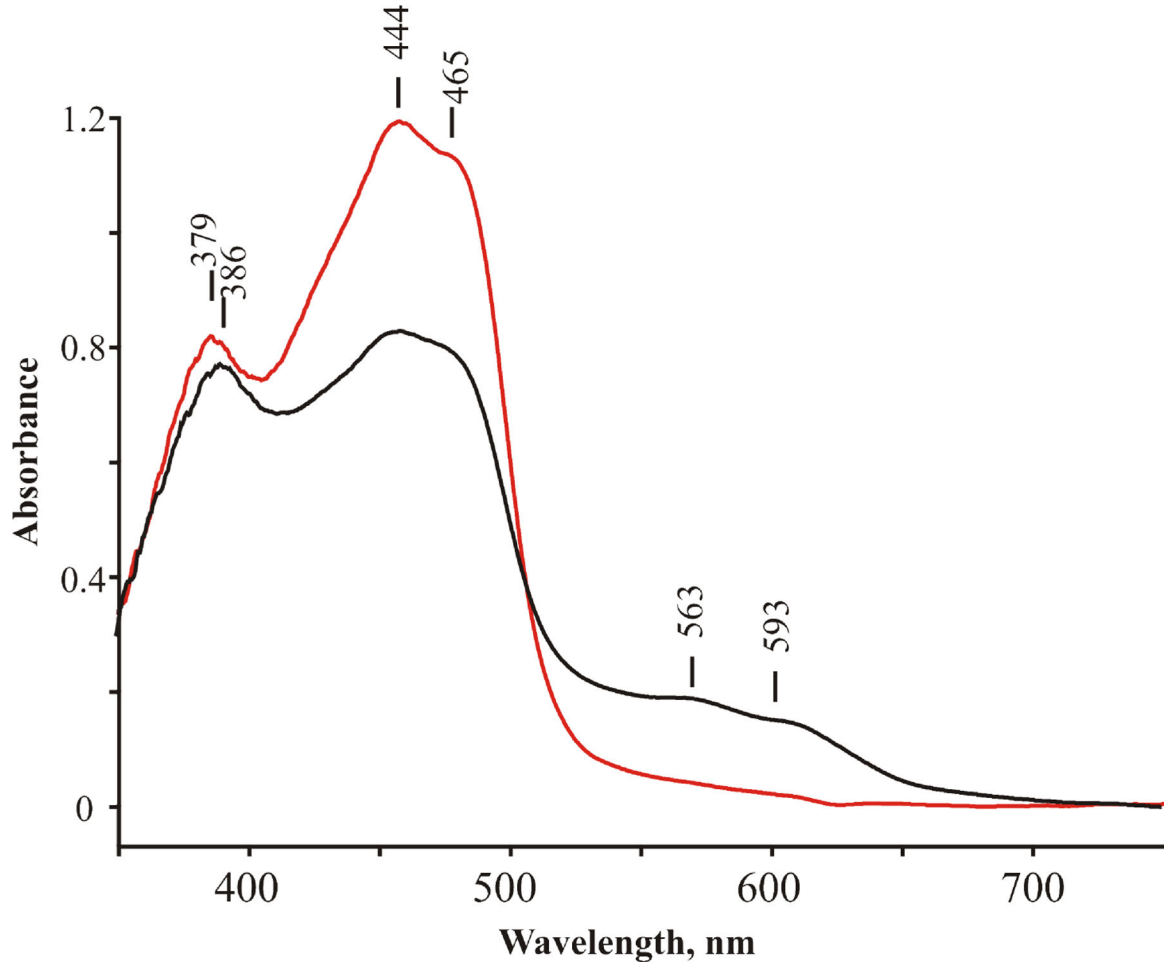

Figure 1.

UV-vis absorbance spectra of 0.4 mM FMN recorded in a transparent glass of 60% glycerol/buffer (pH 7.0) before (red) and after γ-irradiation (black) at 77 K. A dose of 3 Mrad was used in γ-irradiation experiments to generate the spectral traces here and in the other figures.

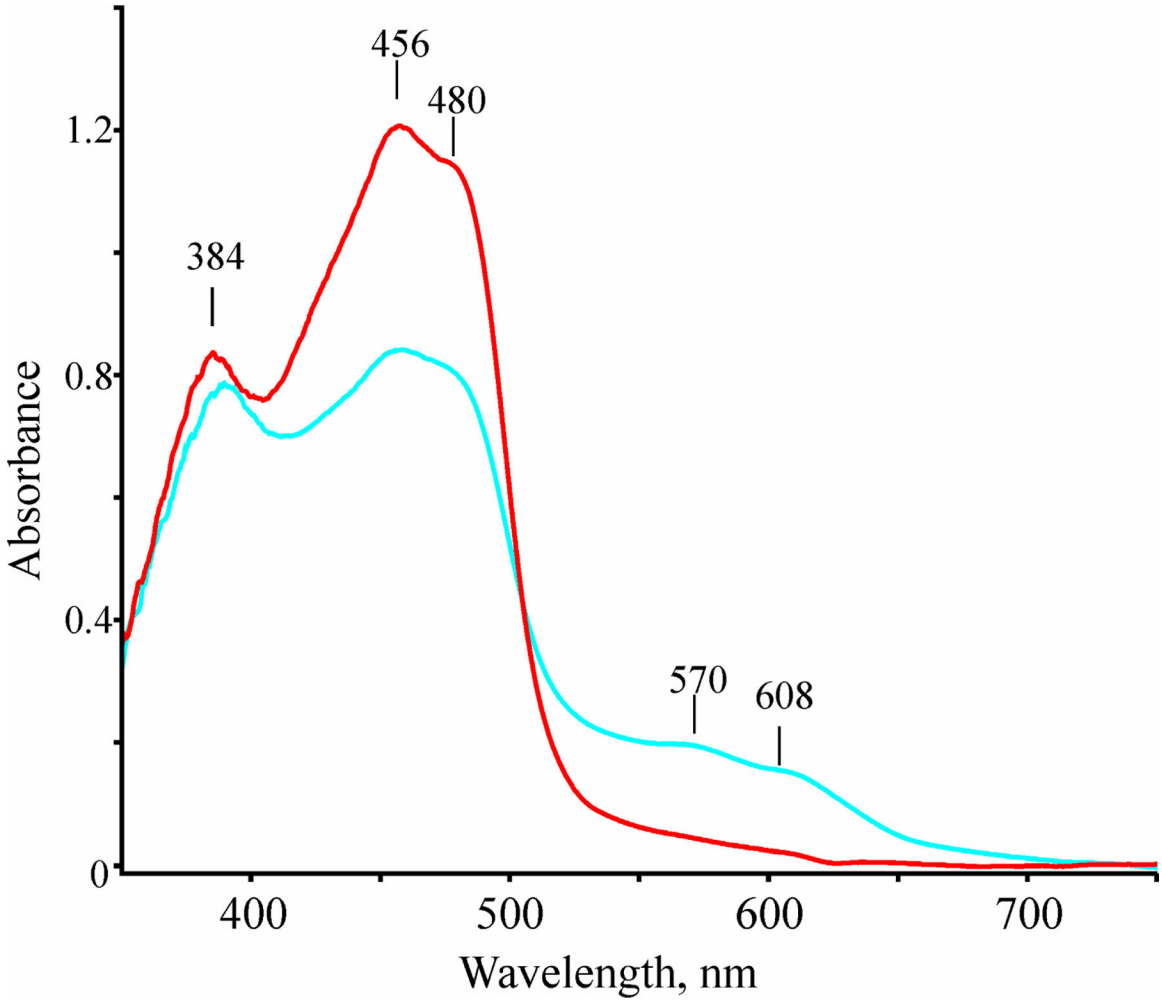

Figure 2.
UV-vis absorbance spectra of 0.4 mM wild type yCPR recorded in a transparent glass of 60% glycerol/buffer (pH 7.0) at 77 K before (red) and after γ-irradiation (cyan).

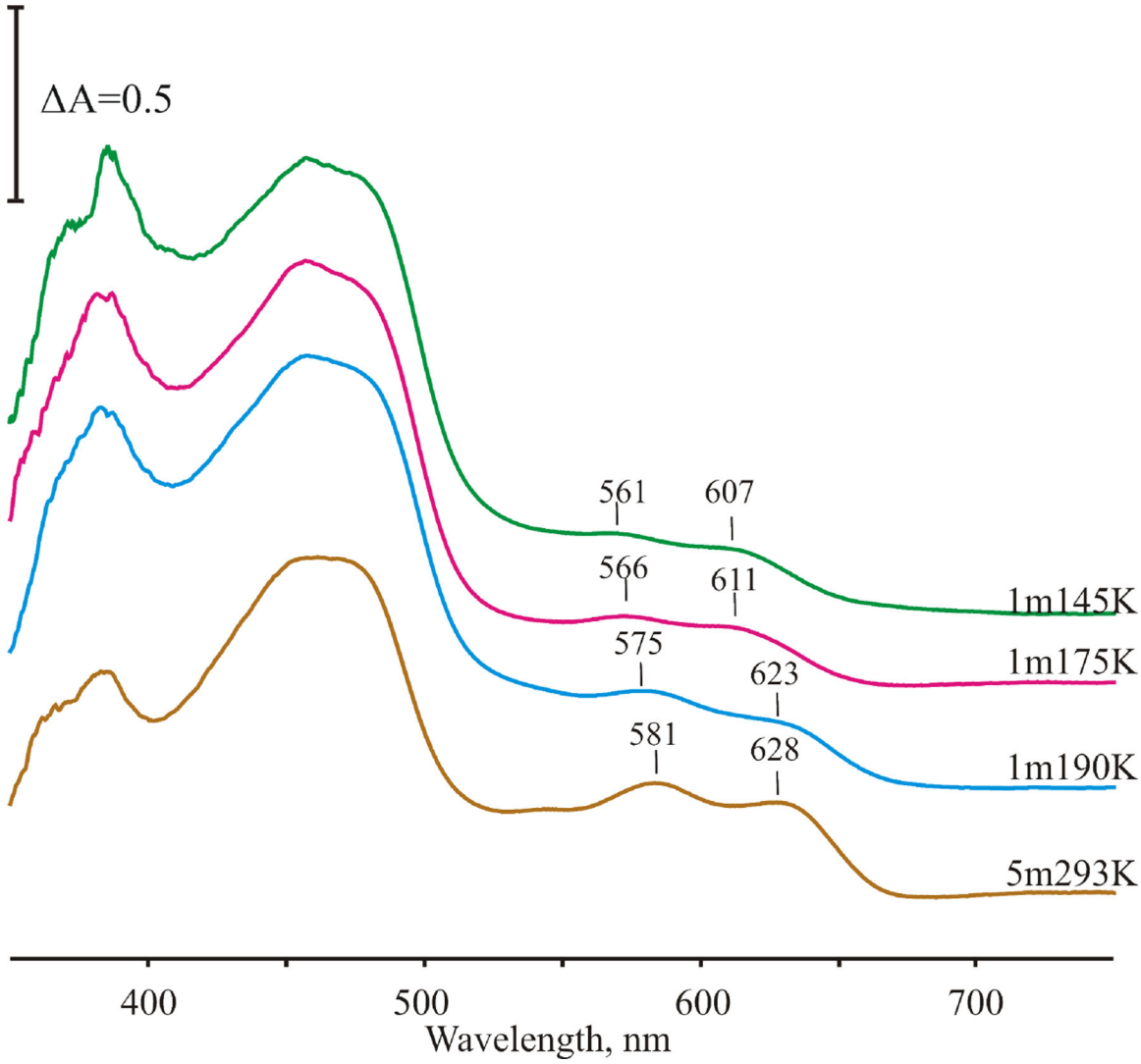

Figure 3.
UV-vis absorbance spectra of 0.4 mM wild type yCPR (radiolytically reduced at 77 K) annealed at indicated temperatures for 1–5 minutes.

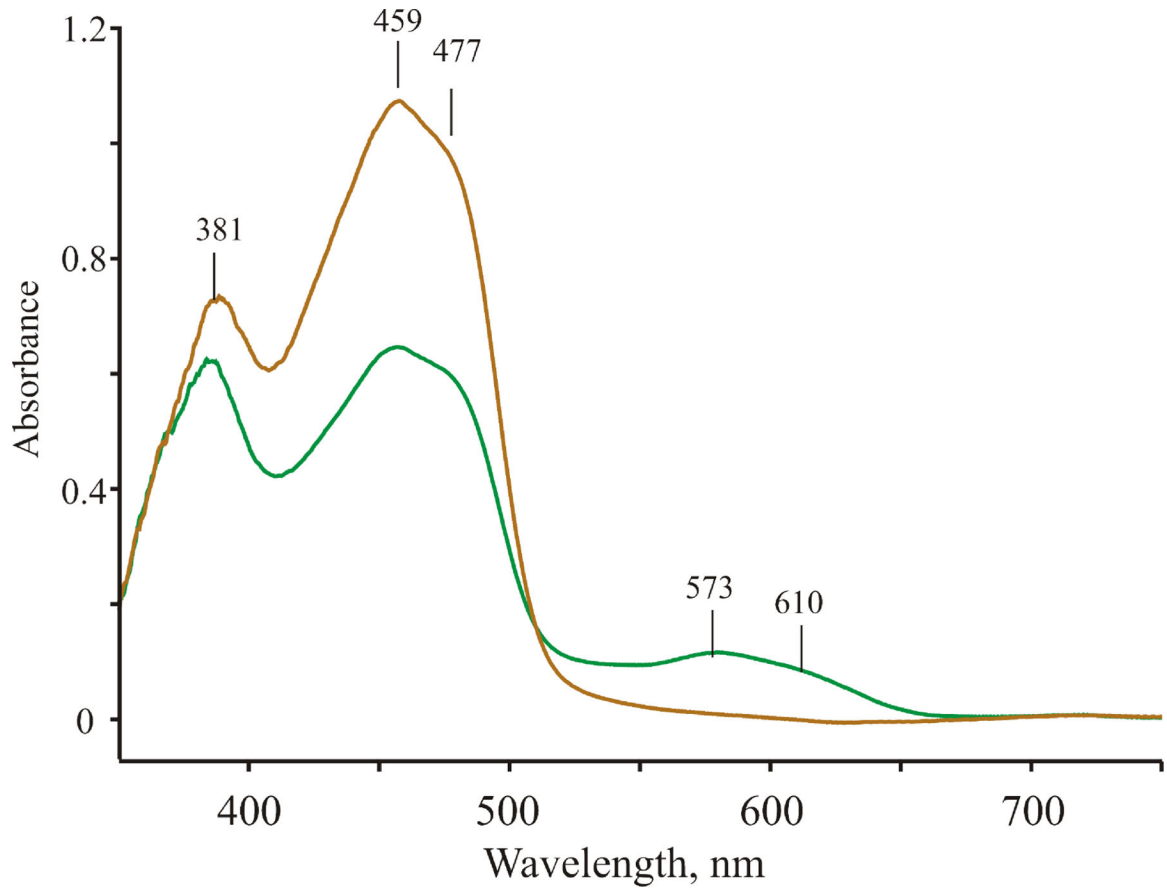

Figure 4.
UV-vis absorbance spectra of 0.4 mM FMN-depleted yCPR recorded in a transparent glass of 60% glycerol/buffer (pH 7.0) before (orange) and after (green) γ-irradiation at 77 K.

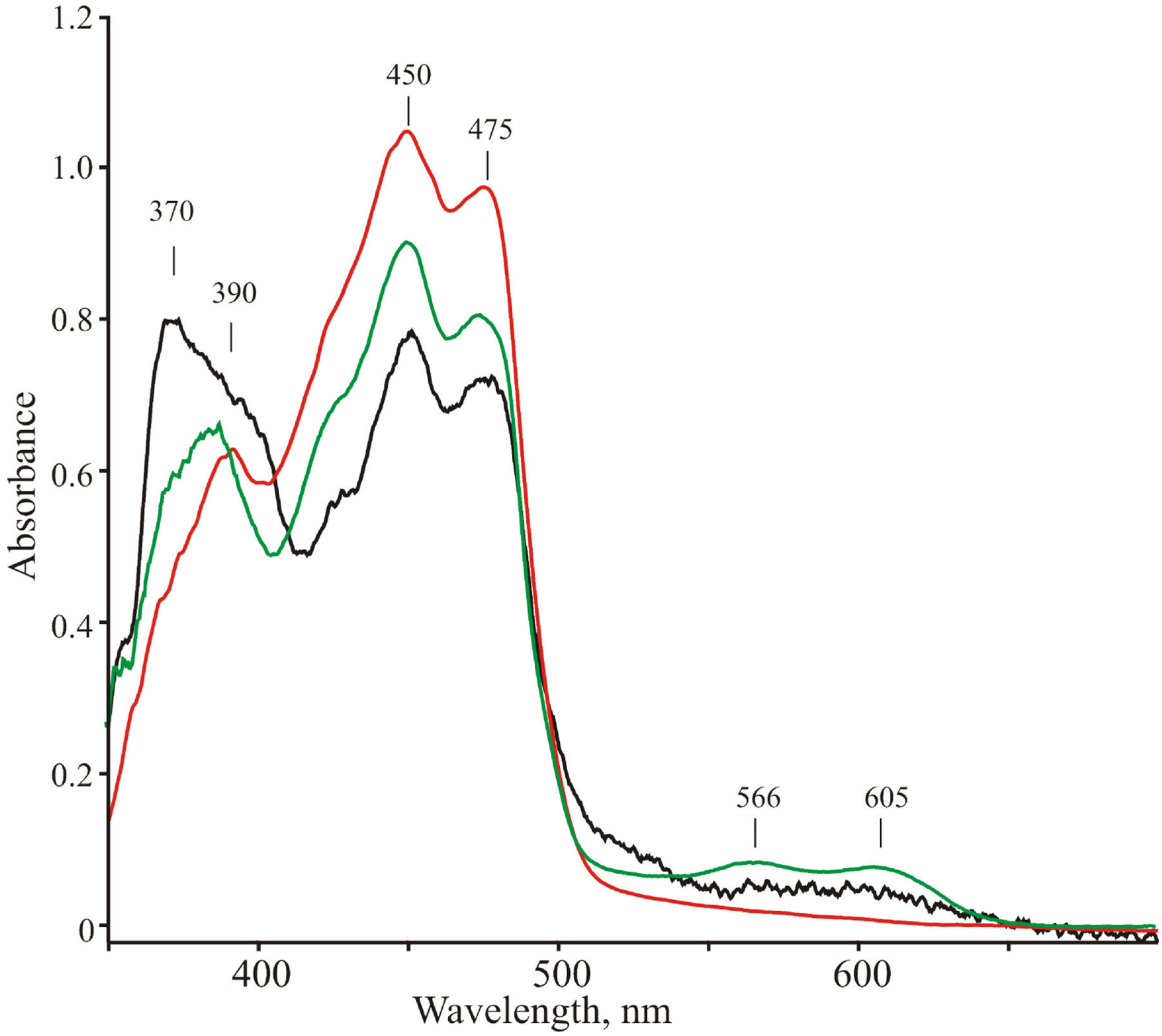

Figure 5.

UV-vis absorbance spectra of 0.4 mM glucose oxidase recorded in a transparent glass of 60% glycerol/buffer (pH 7.5) before (red) and after (black) γ-irradiation at 77 K, followed by annealing at 190 K for 1 min (green).

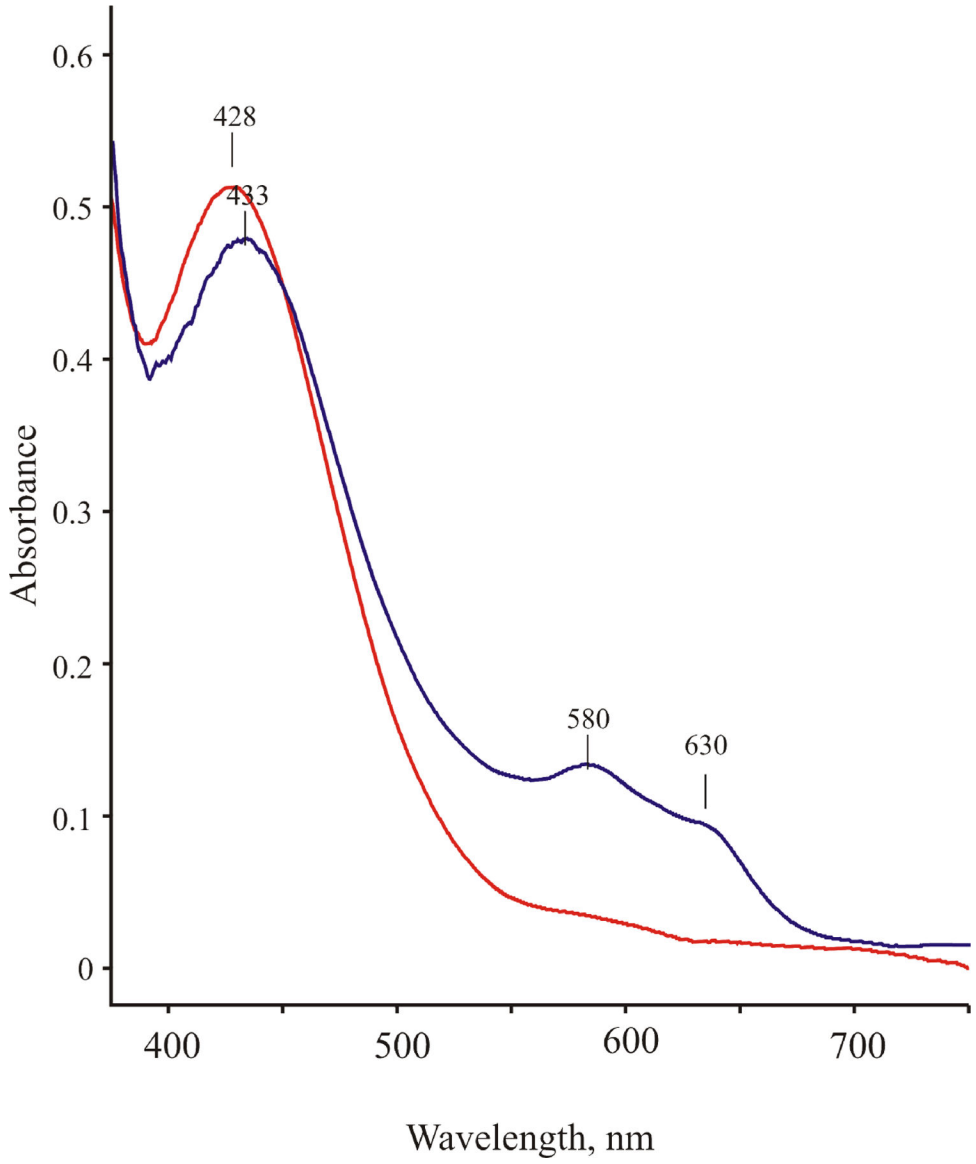

Figure 6.

UV-vis absorbance spectra of 1 mM fully-reduced wild type yCPR recorded in a transparent glass of 60% glycerol/buffer (pH 7.0) before (red) and after (blue) cryo-oxidation by γ-irradiation at 77 K.

Table 1.

Optical parameters of neutral free and CPR-bound FMN and FAD semiquinones.

Species	Temperature,	Absorbance maximum, nm	Absorbance maximum, nm	Absorbance maximum, nm	Reference
Cryo-generated FMNsq	77 K	-	563	593	This work
FMNsq	293 K	481	571	608	[10]
FMNsq	293 K	-	570	620	[20]
FMNsq	293 K	-	570	~620	[20]
Cryo-reduced yCPR	77 K	-	570	607	This work
Relaxed yCPR	77 M	-	581	627	This work
		-			
Air-stable rat CPRsq	293 K	-	580	630	[11, 12]
Cryo-generated FADsq	77 K	-	563	604	This work
FADsq	77 K	-	563	604	This work
Cryo-reduced FMN-depleted yCPR	77 K	-	573	610	This work
Relaxed FMN-depleted yCPR	77 K	-	582	631	This work
Air-stable FAD rCPRsq	293 K	-	~580		[11, 12]
		-			
Cryo-reduced rat CPR	77 K	-	~580		[30]
Cryo-reduced NrdI	77 K	-	570	642	[31]
Air-stable NrdI	293 K		574	608	[31]
Cryo-reduced glucose oxidase, blue semiquinone	77 K	-	565	605	This work
Cryo-reduced glucose oxidase, red semiquinone	77 K	370, ~490			This work
Photoreduced glucose oxidase, blue semiquinone	293 K	-	564	605	[30]
Photoreduced glucose oxidase, red semiquinone	293 K	362, 488			[30]