Cryotrapped Reaction Intermediates of Cytochrome P450 Studied by Radiolytic Reduction with Phosphorus-32*

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Unstable reaction intermediates of the cytochrome P450 catalytic cycle have been prepared at cryogenic temperatures using radiolytic one-electron reduction of the oxy-P450 CYP101 complex. Since a rate-limiting step in the catalytic cycle of the enzyme is the reduction of the ferrous oxygenated heme protein, subsequent reaction intermediates do not normally accumulate. Using 60Co γ-irradiation, the primary reduced oxy-P450 species at 77 K has been identified as a superoxo- or hydroperoxo-Fe(III)-heme complex (Davydov, R., MacDonald, I. D. G., Makris, T. M., Sligar, S. G., and Hoffman, B. M. (1999) J. Am. Chem. Soc. 121, 10654–10655). The electronic absorption spectroscopy is an essential tool to characterize cytochrome P450 intermediates and complements paramagnetic methods, which are blind to important diamagnetic or antiferromagnetically coupled states. We report a method of trapping unstable states of redox enzymes using phosphorus-32 as an internal source of electrons. We determine the UV-visible optical spectra of the reduced oxygenated state of CYP101 and show that the primary intermediate, a hydroperoxo-P450, is stable below 180 K and converts smoothly to the product complex at ~195 K in the course of the thermal annealing, no spectral changes indicating the presence of oxoferryl species (the so-called compound I type spectrum) was observed.

Detailed information about the intermediates in complex chemical and biochemical reactions is of vital importance in mechanistic studies. Typical relaxation methods can often monitor the progress of only one kinetically limiting step of the reaction if there is a dominance of a slow step in the catalytic cycle. It is much more difficult, however, to obtain information about the subsequent (fast) stages of the reaction and the properties of corresponding intermediate species, since they are not accumulated at ambient conditions in the course of the reaction, and their concentrations are nominally very small.

Ideally, one would like to stop the reaction at each step to follow the reaction progress and collect information about each intermediate compound involved in the reaction path. Given a suitable set of activation barriers and enthalpies, cryogenic trapping of unstable intermediates allows such dissection of the reaction cycle, provided a method of preparation of initial nonequilibrium reactive complexes is available. The field of matrix isolation chemistry is based on this approach, where the active complexes are trapped in host matrix, usually solid inert gases at cryogenic temperatures (1). Such methods help to create and study unstable reaction states that rapidly decompose at ambient conditions (2).

Recently, the same approach was developed in field of structural and mechanistic enzymology of the redox active enzyme cytochrome P450 (3–6). The cytochromes P450 are heme-containing metalloproteins involved in numerous biochemical reactions including xenobiotic metabolism and steroid biosynthesis (7). The reaction cycle of these enzymes involves two one-electron reductions, interspersed by the binding of dioxygen. Cleavage of a putative peroxo or reduced oxydioxigen bond is thought to lead to the generation of a high valent “ferryl” intermediate analogous to the compound I state of the peroxidases. The proposed sequential steps of the P450 reaction cycle are depicted in Scheme I,

\[
\begin{align*}
\text{Fe}^{2+}(P) + e^- & \rightarrow \text{Fe}^{3+}(P) \\
\text{Fe}^{2+}(P) + O_2 & \rightarrow \text{Fe}^{3+}(P) - O_2^- \\
\text{Fe}^{2+}(P) - O_2^- + e^- & \rightarrow \text{Fe}^{3+}(P) - O_2^- \\
\text{Fe}^{2+}(P) - O_2^- + H^+ & \rightarrow \text{Fe}^{3+}(P) - (OOH)^- \\
\text{Fe}^{3+}(P) - (OOH)^- + H^+ & \rightarrow \text{Fe}^{4+}(P^{+}+) = O + H_2O
\end{align*}
\]

**SCHEME I**

where (P) represents the porphyrin macrocycle, and (P^{+}+) represents the porphyrin π-cation radical. The sequential one-electron reductions of the heme iron at the active site are provided by a protein redox partner. Alternatively, the same one-electron reduction can be reached by reaction with solvated electrons generated by pulsed radiolysis of water (8). When radiolytic reduction is performed at low temperature (at 77 K), the system is effectively immobilized, and the reactive complexes can be accumulated and studied (3–6).

While these techniques have proved to be important for understanding the detailed chemistry of many redox enzymes (3–6, 9–12), the cryogenic radiolytic reduction remains technically demanding. The high doses (2–6 megarads) that are necessary to obtain sufficient yield of reducing equivalents in frozen solution are usually introduced with powerful 60Co γ-sources or with synchrotron radiation. These radiation sources are not commonly available to biochemical laboratories. Radiolysis at low temperatures (77 K or even 4 K) creates more problems, particularly due to the difficulties in monitoring the progress of the cryoradiolytic reduction in the course of irradiation using these types of sources.

In this paper, we describe how another source of ionizing radiation, phosphorus-32, can be a convenient means for gen-

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erating unstable active compounds through one-electron radiolytic reduction of the reagents trapped in aqueous/organic glass at 77 K. We show that using commercially available $^{32}$P-enriched phosphate it is possible to generate sufficient concentrations of intermediates (radicals or active complexes) of different biological molecules in frozen aqueous/glycerol solutions at 77 K. Although quite unstable at ambient temperatures, many such reaction intermediates are effectively stabilized below the glass transition temperature of solvent and can be accumulated and stored for months (6, 13–15). Different specrotroscopic methods can be used to study the structure of these intermediates and to monitor the progress of subsequent chemical reactions after gradual warming of the system (16–24). Using the technique of *in situ* radiolytic reduction at cryogenic temperature, we present the first determination of the optical spectra of a two electron reduced dioxygen intermediate, the iron-hydroperoxy-oxo state, in cytochrome P450cam (CYP101).

**EXPERIMENTAL PROCEDURES**

$^{32}$P-enriched aqueous solution of orthophosphoric acid (activity 50 mCi/ml) was purchased from Amersham Pharmacia Biotech. Activity was measured by the manufacturer and using the Frick dosimeter method (25). Cytochrome P450 CYP101 from *Pseudomonas putida* was expressed and purified as described (26) and stored in concentrated frozen aqueous solutions at 200 K in the presence of camphor in the ferric form. All other chemicals were of spectrophotometric grade and used without additional purification. Sodium dithionite (Na$_2$S$_2$O$_4$) from Sigma was stored and used in an anaerobic chamber.

Cytochrome P450 CYP101 was reduced by adding several small crystals of dithionite in an anaerobic chamber to a concentrated solution of ferric protein. The products of reaction and the remaining di-thionite were removed by passing the solution through a small G-25 column using deoxygenated 0.1 m potassium phosphate buffer, pH 8.0, containing 1 mM camphor. Solutions of reduced cytochrome P450 were then concentrated using microcentrifuges inside the anaerobic chamber and used within 1 h after preparation.

Samples for incubation with radioactive phosphate were prepared on ice to minimize autoxidation. To prepare the sample, 0.75 ml of the glycerol/phosphate buffer solvent (final camphor concentration 1 mM) was mixed with 0.25 ml of $^{32}$P-enriched orthophosphoric acid, and 60 μl of catalase solution (~1200 units final activity) was added to consume hydrogen peroxide produced due to water radiolysis in radioactive solution occurring during transportation and storage (concentration estimated to be about 1 mM based on radiolytic yield (27)). After incubation of the sample solution for 45 min to eliminate all H$_2$O$_2$, the anaerobic solution of reduced ferrous cytochrome P450 CYP101 (120 μl) was added and stirred for 90 s to ensure the homogeneous mixing with the viscous glycerol solution at 4 °C. The final concentration of the enzyme was 15 μM final concentration of the enzyme prepared in the final glycerol/water mixture at the anaerobic chamber. Both methods gave identical results. The final fraction of autoxidized P450 CYP101 was estimated as less than 5% from absorbance value at 646 nm. The samples were kept in liquid nitrogen and irradiated in a silvered Dewar flask containing liquid nitrogen.

A typical γ-irradiation using $^{60}$Co was for 4 h (at the measured on site dose rate of 21 kilorads/min for a total dose of ~5 megarads), and the irradiation Dewar flask was refilled after 2 h to maintain the samples at 77 K. The similar samples with ferric cytochrome P450, carbon monoxide complex of ferrous cytochrome P450, horseradish peroxidase, and riboflavin were also prepared and irradiated in identical conditions to check the radiolytic reduction yield and compare our results with earlier data (16, 17, 20, 21).

Optical spectra at low temperatures were obtained using a Cary 3 UV-visible spectrophotometer (Varian Instruments) and a homemade cryostat with liquid nitrogen used as a cooling agent. The samples were mixed directly in the disposable methacrylate cells (UV-enhanced semimicro cells from Fisher, total sample volume ~1 ml, 4.3-mm path length, 1.6-mm final optical path length) in the holder of the cryostat. To obtain the good optically transparent frozen solutions without significant turbidity, the 1:1 and 3:1 (v/v) glycerol-buffer solutions were cooled without direct contact with liquid nitrogen to prevent cracking (glycerol and ethylene glycol do not change the catalytic mechanism of CYP 101, although the turnover rate is slowed (28)). As was noted previously (29), the 3:1 glycerol/water mixture is the preferred solvent for all studied temperatures, while 1:1 mixtures undergo phase separation and water crystallization when incubated for some time at 190–230 K, making further optical measurements in the visible region impossible. Below 190 K, both solvents could be used for optical spectroscopy at UV-visible range for prolonged measurements. The temperature was controlled by a calibrated thermostaple fixed in a thermal contact with a brass sample holder close to the light beam position. All spectra were measured in a single beam mode in the 300–900 nm range with 2.0-nm resolution; data points were taken every 1 nm with a scan speed of 60–100 nm/min. Background subtraction, differentiation, singular value decomposition analysis, and all other calculations were accomplished using MATLAB (MathWorks, Natick, MA). In a typical annealing experiment, the sample was warned stepwise, 4–8 K at a rate 0.5–1 K/min. After the several spectra were taken while above the temperature of annealing or 3–5 K below to check the thermal equilibration and reproducibility of the measurements. It was observed that all changes in the spectra of (nonequilibrium) cryoroduced species were irreversible and that partially annealed samples could be cooled again to 77 K and were stable at this temperature for many weeks. Thus, it was possible to work with one sample for several days and to accumulate the products of cyro-oxidation, keeping the sample immersed in liquid nitrogen. The protein integrity after irradiation was tested by UV-visible spectroscopy at the end of the experiment at room temperature. We always obtained a pure CO-bound P450 spectrum with no sign of an inactive form of the protein, P420, being produced. The carbonmonoxy-bound ferrous cytochrome P450 was formed, because the large amount of reducing species and CO formed during radiolysis of aqueous/organic solvent can react with the enzyme when the sample is thawed, as has been observed in previous radiolytic studies (13, 15, 21).

With an increase of the absorbed dose, we observed the appearance and increase of a strong and very broad absorption band with a maximum at about 560 nm, a clear indication of the accumulation of the trapped electrons in the frozen at 77 K aqueous solution within the 1:1 and 3:1 (v/v) glycerol/water mixture. These electrons are photolyzed by the visible light and disappear upon illumination of the sample with the regulated Osram tungsten-halogen lamp with a typical illumination time of 12 min. The cut-off filter (λ > 450 nm) was used to prevent possible degradation of photosensitive intermediates. The sample was fully immersed in liquid nitrogen during illumination to prevent heating. In the separate experiment, it was shown that even with the filter with cut-off A > 600 nm it was possible to eliminate almost all absorption originating from the solvated (aqueous) electrons, although in this case it took longer than 2 h. The possibility of photobleaching this broad background absorption band at the visible region using the light of different spectral composition also confirms its origin as a result of trapped electron accumulation (15, 16, 27). These electrons are photoxyl by the visible light and disappear through recombination via numerous chemical reactions with other products of radiolysis as well as with the original components of the solution.

**RESULTS AND DISCUSSION**

Radioactive $^{32}$P-enriched phosphate is very well suited for use as an internal radiation source in aqueous and organic solutions. Phosphate, as well as sulfate, the product of β-decay of phosphate, are natural components of many buffer systems and do not interfere with most of reactions. The commercially available orthophosphoric acid (as an aqueous or dilute HCl solutions) with $^{32}$P activity up to 50 mCi/ml makes it possible to
reach radiation doses of 30 megarads or more. Accumulation of side radiolysis products, however, usually limits the radiolytic dose to about 5 megarads (24). Since the half-life of $^{32}\text{P}$ is 14.31 days, 2–3 megarads can be generated in a 2-week incubation. This makes easy the monitoring of the progress of radiolytic reduction and accumulation of the primary reaction intermediates, using spectroscopic or other noninvasive methods. A decided advantage of the low temperature, radiolytic trapping of reactive enzyme states is the ability to follow the reaction coordinate of the system by selectively annealing the sample at higher temperatures.

To measure the dose rate generated by $^{32}\text{P}$-enriched phosphate, we used the reaction of aerobic radiolytic oxidation of ferrous sulfate in aqueous sulfuric acid known as the Fricke dosimeter (27). The time course of Fe$^{2+}$ oxidation is monitored by absorption growth at 304 nm (Fig. 1) (25). The agreement between the theoretical curve calculated using the mean energy of electrons generated in $\beta$ decay of $^{32}\text{P}$ (0.7 MeV) and reported activity of the commercial sample and the experimentally measured dose rate is excellent. These results show that $^{32}\text{P}$ can indeed be used as an easy and readily available source of ionizing radiation, successfully replacing $^{60}\text{Co}$ $\gamma$-sources for the radiochemical generation of biological samples in situ.

As has been shown (6, 14, 31, 32), using $\gamma$-irradiation from the $^{60}\text{Co}$ source, it is possible to accumulate the reduced hemoproteins stabilized at 77 K without their conformational or chemical relaxation. Electronic paramagnetic resonance (EPR) (3, 5, 6, 12, 14, 31, 32) and optical (13, 15, 16, 19–21) spectroscopy have been used to study the structure of primary intermediates obtained through radiolytic one-electron reduction of several metalloproteins and the details of subsequent reactions after annealing of the sample at and above the glass transition temperature of the solvent. Here we compare the products of $\gamma$-irradiation at 77 K and of cryoradiolysis using $^{32}\text{P}$ as an internal source and show that both methods give identical results, with each having its own technical advantages.

The radiolytic reduction of cytochrome P450 CYP101 was followed at low temperatures (77 K) in glycerol/water (1:1 or 3:1 (v/v), molar fraction of glycerol $x_g = 0.2$ or 0.43, respectively) frozen solutions containing $^{32}\text{P}$-enriched phosphate containing an activity of 10 mCi/ml, which corresponds to a total dose of $\sim$7 megarads, or 3.5 megarads during the first half-life of $^{32}\text{P}$. Fig. 2A shows the spectra of the oxy form of cytochrome P450 CYP101 immediately after preparation of a sample containing $^{32}\text{P}$ (10 mCi/ml) at 77 K and after incubation at low temperature for different periods of time. The absorbance at 417 nm (maximum of the oxy-P450 spectrum at low temperatures) decreases, and the new peak at $\sim$440 nm appears with time following the increase in absorbed dose. These changes show the progress of one-electron reduction of oxy-P450 and formation of the reduced oxy-P450 complex. To our knowledge, this is a first report of UV-visible spectra of this unstable reaction intermediate of cytochrome P450. This result complements the recent EPR studies of the same system (3, 5). In the EPR measurement, the direct conversion of oxy-P450 to reduced oxy-P450 cannot be observed, since the oxy-P450 is EPR-silent, and only the products of radiolytic reduction can be detected.

The spectra in Fig. 2A were used to estimate the spectrum of the pure reduced oxy-P450 intermediate. Concentration of the remaining oxy-P450 was estimated from these spectra by means of comparing the second derivatives of these spectra. The derivative peaks corresponding to the maxima at 417 and 440 nm are better resolved (results not shown), and hence the area of the former peak was used to calculate the decay in the

![Fig. 1](image1.jpg)

**Fig. 1.** Time dependent aerobic oxidation of ferrous sulfate in the presence of $^{32}\text{P}$ (initial activity 0.25 mCi/ml). Circles, experimental points; full lines, calculated rate of Fe$^{2+}$ oxidation (1) and calculated amplitude (2). Calculations utilized the value of specific activity of radioactive phosphate and the yield of Fricke dosimeter (25, 27), as described under "Experimental Procedures."

![Fig. 2](image2.jpg)

**Fig. 2.** A, optical spectra of oxy-P450 (1) and of radiolytically reduced oxy-P450 at different doses (3–5). Radiolysis is achieved by incubation of the sample of oxy-P450 containing radioactive phosphate (initial activity of $^{32}\text{P}$, 10 mCi/ml) at 77 K for different periods of time. The calculated dose was as follows: 0.9 megarads (2), 2.3 megarads (3), 3.9 megarads (4), 5.1 megarads (5). B, optical spectra of oxy-P450 (1) and of pure reduced oxy-P450 (2) calculated from the spectra plotted in A.
oxy-P450 fraction with increase in dose. The remainder was assigned to the reduced oxy-P450 intermediate, the spectrum of the latter was then calculated using the experimental spectra at Fig. 2A by subtracting corresponding fractions of the oxy-P450 spectrum. The calculated spectrum of the pure reduced oxy-P450 intermediate is shown in Fig. 2B together with the spectrum of pure oxy-P450 shown for comparison. Similar spectra were obtained with the samples of oxy-P450 prepared in 50 and 75% glycerol/water solutions and irradiated with γ-rays from 60Co source with a 4–5-megarad total dose in several independent experiments. This suggests that the radiolytic reduction in frozen solution with solvated electrons acting as reducing species does not depend on the source of the primary radiolytic electrons, γ-photons, or high energy electrons from 32P radioactivity decay. The observed similarity is not surprising, since 32P, 3H, 35S, and other β-active isotopes have been shown to produce the same products of radiolysis in the aqueous solution as do γ-rays (33–36). These results give a solid foundation for generating stable one-electron reduced intermediates at cryogenic temperatures in frozen solutions containing easily obtained β-emitting isotopes.

The spectrum of the reduced oxy-P450 shown in Fig. 2B is in excellent agreement with that calculated by Harris et al. (37) for the same system. The pronounced split Soret band and 30-nm red shift were the main features of their calculated spectrum. Our experimentally obtained red shift is 23 nm, and the split Soret shape of the spectrum is remarkably similar to the theoretical results. Optical changes have also been observed using pulse radiolysis of oxycomplex of the deuteroheme-substituted cytochrome P450 CYP101 (8), which are nearly identical to our results on the native metalloprotein containing protoporphyrin IX as a prosthetic group.

The irreversible evolution of the reduced oxy-P450 reaction intermediate with temperature increase was monitored by the spectra shown in Fig. 3. The gradual annealing of the samples reduced radiolytically at 77 K results in the sequence of conformational relaxations and chemical transformations. At the same time, the various organic radicals, which appear as the products of glycerol radiolysis, gradually recombine and decay. These latter processes result in continuous changes of the background absorbance in the optical spectra in the visible and near UV range. To subtract this background, the spectra of the 75% glycerol/buffer solution without added cytochrome P450 were obtained. The reference sample was irradiated in identical conditions (total dose 5 megarads) and carefully annealed from 77 up to 240 K. In these experiments, spectra were taken every 4 K at temperatures above 140 K, where significant optical changes begin to occur. The resulting spectral array was used for background subtraction in the analysis of the enzyme spectra at different temperatures with the actual base line at each temperature calculated using linear interpolation with respect to the temperature.

The calculated spectra of pure reduced oxy-P450 with subtracted base line as described are shown in Fig. 3A in a three-dimensional representation. The simple visual inspection of these spectra shows only one main process, the decay of the primary reaction intermediate with a Soret maximum at 443 nm and concomitant increase in absorbance at 392 nm, a characteristic of the high spin ferric cytochrome P450. More careful analysis reveals two minor temperature-dependent spectral processes. The presence of only four spectrally distinguishable components was confirmed by singular value decomposition analysis (data not shown). All spectral changes are observed in the temperature interval 192–205 K and are presented in the more convenient form in Fig. 3B.

The first process is the red shift of the maximum from 440 to 443 nm in the narrow temperature interval 192–194 K, which is not accompanied by notable optical changes in other regions of the spectrum. This small shift could be due to protonation of superoxide anion bound to iron of the heme (5), to thermal relaxation of the heme-ligand reduced complex, or to other relatively minor perturbation of the chromophore. At the same temperature, the beginning of the product formation is observed with the active reduced oxy-P450 intermediate decay. The amplitude of the peak at 443 nm decreases, the new peak appears at 417 nm, and the absorbance at 392 nm begins to increase. The Soret peak at 417 nm is characteristic of the low spin ferric cytochrome P450, the state at which the heme iron is hexacoordinated with the weak sixth ligand. In the CYP101 system, the appearance of this maximum can be assigned to the formation of the product-bound ferric cytochrome P450, the oxygen of the hydroxyl group in 5-exo-hydroxycamphor being coordinated with heme iron as a sixth ligand. The formation of this complex as an intermediate step before the product release, obtained at the same temperature in aqueous/glycerol solution was also determined by EPR spectroscopy (5), and our optical data confirm this observation. The longer annealing at slightly higher temperatures results in the disappearance of the signal from this low spin product complex and an increase in absorption.
Intermediate States of Cytochrome P450

of absorbance at 392 nm from high spin ferric cytochrome P450.

The stabilization and detection of the main putative active intermediate in cytochrome P450, proposed to have the main features of the “compound I” state of peroxidases, the oxoferryl porphyrin $\pi$-cation radical (38, 39), remains a subject of debate. In radiolytic reduction and annealing experiments, we did not obtain any spectral evidence for the existence of this state. The only new intermediate observed is the peroxo (hydroperoxo) complex. The known features of a compound I-type spectra, namely a broad Soret maximum between 370 and 410 nm with relatively low amplitude and an increase of absorbance at 650–700 nm (40), were not observed. This may indicate that even at 200 K the active intermediate has high activity and is not accumulated at sufficient concentrations. Alternatively, the lack of the aforementioned features in visible spectra can be the result of the different electronic structure of the active intermediate in cytochrome P450 compared with the known analogous in chloroperoxidase, horseradish peroxidase, and model systems. Such a difference may involve, but certainly not be limited to, the different distribution of unpaired electron density due to the presence of thiolate proximal ligand (39, 41–43). An alternative explanation involving a different active hydroxylation compound has been proposed by Newcomb and Toy (44).

In radiolytic reduction and annealing experiments, we did not observe the formation of ferric P450 and the product (5). The electron reduced oxy-P450 is stable at low temperatures (77–190 K) and undergoes a series of irreversible transformations resulting in formation of ferric P450 and the product (5). The one-electron reduced oxy-P450 is stable at low temperatures (77–190 K) and undergoes a series of irreversible transformations resulting in formation of ferric P450 and the product (5). The single oxygen-containing intermediate in CYP101. Such an intermediate was obtained by means of EPR and ENDOR analysis of H/D exchangeable protons in all reaction intermediates (5), although the direct observation of compound I was not achieved. However, using x-ray crystallography, Schlichting et al. (4) observed evidence for the transient formation of a single oxygen-containing intermediate in CYP101. Such an observation may be due to further stabilization of highly reactive intermediates by the crystal lattice or subtle differences in...