Rapid P450 Heme Iron Reduction by Laser Photoexcitation of Mycobacterium tuberculosis CYP121 and CYP51B1

ANALYSIS OF CO COMPLEXATION REACTIONS AND REVERSIBILITY OF THE P450/P420 EQUILIBRIUM

Received for publication, April 9, 2007, and in revised form, May 25, 2007. Published, JBC Papers in Press, June 6, 2007, DOI 10.1074/jbc.M702958200

Adrian J. Dunford, Kirsty J. McLean, Muna Sabri, Harriet E. Seward, Derren J. Heyses, Nigel S. Scrutton, and Andrew W. Munro

From the ‡Manchester Interdisciplinary Biocentre, Faculty of Life Sciences, University of Manchester, 131 Princess Street, Manchester M1 7DN and the §Department of Biochemistry, University of Leicester, Henry Wellcome Building, Lancaster Road, Leicester LE1 9HN, United Kingdom

We demonstrate that photoexcitation of NAD(P)H reduces heme iron of Mycobacterium tuberculosis P450s CYP121 and CYP51B1 on the microsecond time scale. Rates of formation for the ferrous-carbonmonoxo (FeII-CO) complex were determined across a range of coenzyme/CO concentrations. CYP121 reaction transients were biphasic. A hyperbolic dependence on CO concentration was observed, consistent with the presence of a CO binding site in ferric CYP121. CYP51B1 absorption transients for FeII-CO complex formation were monophasic. The reaction rate was second order with respect to [CO], suggesting the absence of a CO-binding site in ferric CYP51B1. In the absence of CO, heme iron reduction by photoexcited NAD(P)H is fast (10,000–11,000 s⁻¹) with both P450s. For CYP121, transients revealed initial production of the thiolate-coordinated (P450) complex (absorbance maximum at 448 nm), followed by a slower phase reporting partial conversion to the thiol-coordinated P420 species (420 nm). The slow phase amplitude increased at lower pH values, consistent with heme cysteinate protonation underlying the transition. Thus, CO binding occurs to the thiolate-coordinated ferrous form prior to cysteinate protonation. For CYP121, slow conversions of both the ferrous/FeII-CO forms to species with spectral maxima at 423/421.5 nm occurred following photoexcitation in the absence/presence of CO. This reflected conversion from ferrous thiolate- to thiol-coordinated forms in both cases, indicating instability of the thiolate-coordinated ferrous CYP121. CYP121 FeII-CO complex pH titrations revealed reversible spectral transitions between P450 and P420 forms. Our data provide strong evidence for P420 formation linked to reversible heme thiolate protonation, and demonstrate key differences in heme chemistry and CO binding for CYP121 and CYP51B1.

‡The work was supported in part by the United Kingdom Biotechnology and Biological Sciences Research Council (BBSRC) and European Union FP6 Project NM4TB. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

BBSRC professorial fellow.

To whom correspondence should be addressed. Tel.: 44-161-306-5151; Fax: 44-161-306-8918; E-mail: Andrew.Munro@manchester.ac.uk.

© 2007 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.

The abbreviations used are: Mtb, Mycobacterium tuberculosis; CYP, cytochrome P450.
ferric, low-spin enzyme) to ~450 nm (for the FeII-CO complex) is a hallmark of the P450s and is associated with a thiolate (cysteinyl) proximal ligand to the heme iron (Cys345 in Mtb CYP121, Cys194 in Mtb CYP51B1). Protonation of the thiolate to a thiol is associated with a Soret absorption shift to ~420 nm for the FeIII-CO complex (18). This “P420” form is commonly considered an inactivated form of P450 enzymes, and can be formed by disrupting enzyme structure using, e.g. chaotropic reagents (19). However, recent studies of the Sorangium cellulosum P450epok (CYP167A1) indicate that the time-dependent conversion of the P450 FeII-CO complex to the P420 form can be reversed in the presence of the substrate epothilone D, leading to “substrate-mediated rescue” of the inactivated protein and formation of substantial amounts of the P450 form with an absorbance maximum at 446 nm (20). Studies of CYP51B1 also showed that an irreversible time-dependent conversion of P450 to P420 occurred following FeII-CO complex formation, but this process could be dramatically slowed if the protein was bound to a substrate analogue (estriol) (16). The FeII-CO complex of CYP121 (formed by bubbling CO into a solution of sodium dithionite-reduced enzyme) also displays a substantial component of P420 at neutral pH, although the P450/P420 FeII-CO ratio is quite stable following complex formation in this case.

Recently, we exploited laser photoccitation of the Bacillus megaterium P450 BM3 (CYP102A1) enzyme on a microsecond time scale, and to measure kinetics of CO binding to the protein (21). We have now exploited this method to analyze the kinetics of P450 reduction, FeII-CO complex formation, and the P450 to P420 collapse in the Mtb CYP51B1 and CYP121 P450s. In this work, we demonstrate, for both enzymes, that the P450 FeII-CO complex is formed rapidly on laser photoccitation of the oxidized enzyme in the presence of CO. This is followed by a slower process of conversion to P420, demonstrating that the heme thiolate protonation is a consequence of heme iron reduction and/or CO coordination, and that there is no significant amount of thiol coordination of ferric heme iron in the resting forms of either P450. Markedly different kinetic properties and CO concentration dependence was observed for CYP121 and CYP51B1 FeII-CO complex formation, suggesting distinctive modes of CO association with these enzymes. We also demonstrate that the CYP121 P450/P420 equilibrium is reversible and exquisitely pH-dependent, reinforcing the importance of thiolate protonation in the formation of the cytochrome P420 species.

**EXPERIMENTAL PROCEDURES**

**Expression and Purification of CYP121**—The Mtb Rv0764c gene encoding the CYP51B1 was also expressed in E. coli strain HMS174(DE3) using a PET20b (Novagen) plasmid system, as described previously (16). CYP51B1 purity was established by SDS-PAGE analysis, and from the Soret A416:A280 ratio for the purified ferric enzyme. An A416:A280 ratio of ~1.8 indicated pure CYP51B1. Purified CYP121 was concentrated by ultrafiltration (to >500 μM, using a Centriprep 30), dialyzed into 10 mM Tris-HCl (pH 7.5), and stored at ~80 °C until required. CYP51B1 concentration was determined using ε419 = 134 mM−1 cm−1 for the oxidized enzyme, as described previously (16).

**Laser Flash Excitation of CYP121/CYP51B1**—Prior to use in laser excitation experiments, CYP51B1 and CYP121 samples were exchanged into buffer A by passage through a PD-10 gel filtration column pre-equilibrated with anaerobic buffer A. Buffers were made anaerobic by extensive bubbling with oxygen-free nitrogen, prior to incubation in an anaerobic glove box (Belle Technology, Portesham, UK), with oxygen levels maintained at <2 ppm. All laser excitation experiments were done in sealed, air-tight quartz cuvettes. For laser photoexcitation experiments, 1 ml samples of CYP121 or CYP51B1 enzymes (typically 3–6 μM in buffer A for data collection over time bases of >1 ms, or 10 μM for data collection over time bases of <1 ms) were excited using the 3rd harmonic (355 nm) of a Q-switched Nd-YAG laser (Brilliant B, Quantel) in a sealed quartz cuvette of 1-cm path length. The energy output of each laser pulse was up to 200 millijoules and pulses were 6–8 ns in duration. Other components of the various reaction mixtures used were pyridine nucleotide coenzymes (NADH or NADPH in the range from 0 to 500 μM), the CYP51B1 substrate analogue epothilone A (used at a final concentration of 50 μM; higher concentrations were avoided to optimize data quality) (16), and carbon monoxide (CO, used across the concentration range from 0 to 975 μM, where the latter is saturating in solution at atmospheric pressure and 298 K) (22). Spectral transients were recorded at a variety of wavelengths to follow coenzyme oxidation, CYP121 and CYP51B1 heme iron reduction and FeII-CO complex formation. These included 340 (Δε340 = 6210 M−1 cm−1 for the oxidation of NAD(P)H to NAD(P)+), 416–419 (near the Soret absorbance maximum for low-spin CYP121 and CYP51B1), 410 (at the Soret peak for ferrous CYP121), 423 (at the Soret maximum for reduced, thiol-coordinated CYP51B1) (Fig. 1A), 550 and 559 (to monitor changes in the visible bands of the P450 hemes), and 450/420 nm (to follow formation/decay of nyl-Sepharose, Q-Sepharose, and hydroxyapatite resins according to established protocols (11). Pure CYP121 was concentrated by ultrafiltration (Centriprep 30, Millipore) and exchanged into 100 mM potassium phosphate (potassium Pi, pH 7.0) (buffer A) plus glycerol (50% v/v) by dialysis, prior to storage of pure P450 at ~80 °C. Purity of CYP121 was assessed by spectral properties (ratio of Soret absorption at 416.5 nm to protein-specific absorption at 280 nm, with an A416.5:A280 ratio of >1.8 indicating pure protein), and by SDS-PAGE analysis of protein samples (on 10% denaturing gels). CYP121 concentration was determined from the Soret absorption of the ferric enzyme in its ligand-free low-spin state using ε416.5 = 95 mM−1 cm−1, as described previously (11).
P450 Heme Iron Reduction of CYP51B1 and CYP121

the corresponding Fe^{III}-CO complexes). Development of a peak at \(-450 \text{ nm}\) is characteristic of native (thiolate-coordinated) Fe^{III}-CO complexes of the P450s, where \(A_{450-490} = 91,000 \text{ M}^{-1} \text{ cm}^{-1}\) for the difference spectrum generated by subtraction of the spectrum for ferrous [Fe^{II}P450 from that for its Fe^{III}-CO complex (23). Parallel experiments were performed for enzyme samples in the presence and absence of CO. Data collection was made using an Applied Photophysics LKS-60 flash photolysis instrument with a detection system at right angles to the incident laser beam. The probe light (150 watts xenon lamp) was passed through a monochromator before and after passage through the sample. Absorbance changes were measured using a photomultiplier tube and kinetic transients were typically collected from 50 ms up to 10 s. Photodiode array spectral data were also collected for slow reaction phases, and for data reporting on the conversions between P450 and P420 Fe^{II}-CO enzyme forms. Multiple kinetic traces for each sample were measured and averaged prior to data analysis. Optical spectra were recorded before and after laser photoexcitation to ensure protein integrity. Transients were recorded at 25 °C.

For measurements over faster time scales (typically <1 ms), the output of the xenon arc lamp was pulsed using a xenon arc pulser (Applied Photophysics) and transients were measured using an Infiniium oscilloscope model number 54830B (Agilent Technologies). Absorbance spectra were measured before and after laser photoexcitation by transferring the cuvette to a Cary 50 UV-visible spectrophotometer (Varian) and recording data between 300 and 800 nm.

All other spectral measurements for enzyme quantification and for establishing features of CYP51B1 and CYP121 in ferric substrate-free and substrate-bound forms, the ferrous state, and in the Fe^{III}-CO complex were also performed using a Cary 50 UV-visible spectrophotometer, either on the bench or under anaerobic conditions in a glove box (Belle Technology, Portesham, UK) for the ferrous enzymes.

Data from photoexcitation experiments were analyzed and fitted to appropriate exponential functions using the manufacturer’s (Applied Photophysics) kinetics analysis software. All spectral and single wavelength data were transferred to Origin (OriginLab, Northampton, MA) for further analysis. Absorption transients accompanying formation of the Mtb CYP121 Fe^{III}-CO complexes were fitted accurately to a standard double exponential function, and rates for each phase (\(k_{\text{obs}}\)) were plotted versus the concentration of other ligands/substrates varied for any set of experiments (CO or NAD[P]H). In the case of Mtb CYP121, reaction transients were fitted accurately by a single exponential function and were plotted as for CYP121. For experiments in which CO concentration was varied, a hyperbolic dependence of the Fe^{III}-CO complex formation rate on CO concentration was observed in the case of CYP121. Data were fitted to a hyperbolic function to obtain estimates for the apparent limiting rate constant for Fe^{III}-CO complex formation (\(k_{\text{lim}}\)), and for the apparent dissociation constant for the Fe^{III}-CO noncovalent complex. In the case of CYP121, a linear dependence of rate constant on CO concentration was obtained and data were fitted accordingly.

A pH titration to examine the conversion of the CYP121 Fe^{III}-CO complex between P450 and P420 forms was performed by measurement of the absorption spectrum of CYP121 (5.6 \(\mu\text{M}\)) in 100 mM potassium Pi, with pH titrated between 6.5 and 10.5 using potassium hydroxide. To show the reversibility of the protonation of the Cys^{345} thiolate, HCl was added to lower the pH again to 6.5. The reversibility of the P450/P420 transition in this pH range demonstrated that the enzyme and its heme binding site remained intact throughout the titration.

Materials—Bacterial growth media (Tryptone, yeast extract) were from Melford Laboratories (Ipswich, Suffolk, United Kingdom). All other reagents were from Sigma (Poole, Dorset, UK) and were of the highest grade available.

RESULTS

Laser Photoexcitation of CYP121—Spectral features of the Mtb CYP121 enzyme in its oxidized (Fe^{III}), sodium dithionite reduced (Fe^{II}), and reduced/carbon monoxide-bound (Fe^{II}-CO) forms are shown in Fig. 1B. The ferric enzyme is predominantly low-spin with its Soret band at 416.5 nm. EPR spectroscopy indicated that the ferric enzyme was thiolate coordinated with no sign of any cysteine thiol-coordinated (P420) form (11). Reduction leads to a shift of the Soret feature to 405 nm, con-
observed recently for the P450 BM3 enzyme (21). As shown in Fig. 2B, there was a hyperbolic dependence of Fe^{II}-CO complex formation rate on the concentration of CO in the solution (in the range from 0 to 975 μM, where the latter is saturating at 25 °C). There was no apparent dependence of Fe^{II}-CO complex formation rate on the coenzyme concentration, with rates determined at individual concentrations of CO being indistinguishable (within error) for NADH and NADPH in the range from 50 to 500 μM. The observed rate constants for formation of the Fe^{II}-CO complex (at pH 7.0) at various NAD(P)H concentrations were fitted to a hyperbolic function to obtain limiting rate constants $k_{\text{lim}} = 1887 \pm 164$ s$^{-1}$ (NADPH) and $1648 \pm 116$ s$^{-1}$ (NADH) from the data for the fast phase of the biphasic reaction transients. The corresponding apparent $K_d$ values for CO were $914 \pm 139$ μM (with NADPH) and 760 ± 100 μM (with NADH). The slower phases also showed a hyperbolic rate dependence on CO concentration (Fig. 2B). The relatively weak affinity for CO and hyperbolic dependence observed are likely explained in terms of a low affinity site for the CO in the ferric enzyme matrix (see Ref. 21 for detailed discussion). Following reduction of the iron to the ferrous state on laser photoexcitation of NAD(P)H, the CO relocates to the higher affinity site on the heme iron, to form the Fe^{II}-CO complex. The biphasic nature of the reaction transients might be explained by the presence of two conformations of the CYP121 protein, or else distinct binding sites for CO in the ferric enzyme. Structural studies of CYP121 indicate a form with a constricted active site, and conformational alterations are expected to facilitate substrate binding and catalysis (12, 13).

In view of the predominance of the P420 form of the CYP121 Fe^{II}-CO complex at neutral pH, as evident from spectral analysis (Fig. 1B), we analyzed the evolution of the entire absorption spectrum of the P450 following laser photoexcitation of CYP121 to form the Fe^{II}-CO complex (Fig. 3A). The data collected demonstrated clearly that the first species formed has absorption maximum at 448 nm (i.e. the P450 form), but that this collapses progressively over a period of several seconds, with concomitant formation of a species with absorption maximum at 420 nm (i.e. the P420 form). Thus, the data indicate that the thiolate-coordinated P450 Fe^{II}-CO complex forms first following CYP121 heme reduction by laser photoexcitation of NAD(P)H in the presence of CO, but that this species is in equilibrium with the thiol-coordinated P420 form. The balance lies in favor of the P420 form at neutral pH, and this form predominates when the system comes to equilibrium. The rate of the P450 to P420 decay process is $0.58 \pm 0.06$ s$^{-1}$ at pH 7.0 and 25 °C. The apparent decay rate is not substantially altered at other pH values examined.

**Laser Photoexcitation of CYP51B1**—The spectral features of the Mtb CYP51B1 enzyme in its Fe^{III}, Fe^{II}, and Fe^{II}-CO forms are shown in Fig. 1A. As with CYP121, CYP51B1 is predominantly low-spin in its ferric state, with Soret maximum at 419 nm. EPR data also demonstrated a thiolate-coordinated P450 heme iron in the substrate-free enzyme (4, 16). However, the Fe^{II}-CO complex collapses from the P450 to a P420 form over a period of minutes, and the Fe^{II} form also has a spectrum typical of a thiol-coordinated heme iron and comparable with thiol-coordinated heme iron in a H93G mutant of sperm whale myo-
globin (3, 4, 16). Thus, there are considerable differences between the properties of the two Mtb P450s CYP121 and CYP151B1.

To investigate kinetics of formation of the CYP51B1 Fe\(^{II}\)-CO complex and to compare the efficiency of the process with that of the CYP121 enzyme, laser photoexcitation experiments were done as described above for CYP121. As with CYP121, we observed a rapid absorption increase at 450 nm, indicative of the formation of the thiolate-coordinated Fe\(^{II}\)-CO complex of CYP51B1, using either NADH or NADPH as electron donor. In addition, it was established that there was negligible dependence of the rate of the Fe\(^{II}\)-CO complex formation with NADPH or NADPH in the range from 50 to 500 \(\mu M\) coenzyme. However, the absorption transients obtained for CYP51B1 (and with either coenzyme as reductant) were monophasic and fitted accurately to a single exponential expression. Fig. 4A shows a typical transient collected at 450 nm for CYP51B1, with data fitted to a single exponential function. The dependence of the formation of CYP51B1 Fe\(^{II}\)-CO complex rate on CO concentration was also distinct from that observed for CYP121, with a second order rate constant of 0.185 ± 0.021 \(\mu M^{-1} s^{-1}\). Data were essentially identical with NADPH or NADH as electron donor. In the estriol-bound (50 \(\mu M\)) form, transients were monophasic and the plot of \(k_{obs}\) versus [CO] remained linear (shown as filled triangles), with a slightly shallower gradient (second order rate constant of 0.134 ± 0.019 \(\mu M^{-1} s^{-1}\) and with NADPH (300 \(\mu M\)) as electron donor. The \(k_{obs}\) intercept is at 0.4 s \(^{-1}\) in both cases, suggesting a slow CO off-rate for the Fe\(^{II}\)-CO complex.
CYP121) rates obtained at various CO concentrations were virtually identical for NADPH and NADH. The rate of FeII-CO complex formation in CYP51B1 was slower than that observed in CYP121. For example, at saturating CO and using NADPH at 300 μM, the apparent rate was $\sim 180$ s$^{-1}$. At a similar concentration of NADPH and CO, the respective CYP121 FeII-CO complex formation rate was $>900$ s$^{-1}$. On binding the substrate analogue estradiol (and with NADPH as electron donor), reactions remained monophasic, and the reaction rate dependence on CO concentration remained linear. The second order rate constant was slower at 0.134 s$^{-1}$. The transient kinetic data are consistent for CYP51B1 and that there is a second order dependence of FeII-CO complex formation rate on CO concentration might be explained in terms of the absence of any relevant CO binding site for the ligand in the ferric enzyme. Thus, CO has significant affinity only for the ferrous iron formed in CYP51B1 following laser excitation.

**Kinetics of the CO Collapse Process in CYP121 and CYP51B1**—As discussed above, the equilibrium position of the P450-P420 complex lies considerably toward the P420 form for the CYP121 species formed by dithionite reduction at neutral pH. We examined the evolution of the spectral properties of CYP121 at pH 7.0 following laser excitation in the presence of saturating CO and NADPH (300 μM). We observed (using a photodiode array attachment on the laser apparatus) a substantial (but not complete) collapse of the P450 species to P420 over a period of $\sim 10$ s. In view of protonation of cysteine thiolate likely underlying the formation of P420 in CYP121, we examined the decay of P450 to P420 across a range of pH values and with single wavelength analysis at 450 nm. As seen in Fig. 3B, at $pH 9$ there was only a very small amount of formation of the P420 form of CYP121. However, as pH was lowered there was progressively more P420 formation, consistent with the proposed role of thiolate protonation in the formation of the P420 species. Rates of decay from single wavelength data were consistent with the values determined from PDA analysis, at $0.72 \pm 0.02$ s$^{-1}$.

As has been observed previously, the FeII-CO complex of CYP51B1 collapses from the P450 form to the P420 form over a period of several minutes (3, 16). At pH 7 and 25 °C, the rate was re-determined by following the spectral collapse and fitting data to a single exponential function. A rate of ~0.25 min$^{-1}$ was determined, consistent with previous data (16).

**A Reversible P450/P420 Equilibrium in CYP121**—In view of the substantial effects of pH on the final P450/P420 balance in kinetic experiments with CYP121, we decided to evaluate the P450/P420 equilibrium by static pH titration. As shown in Fig. 5 (and consistent with results from laser excitation experiments), the equilibrium is exquisitely poised, with almost complete P450 stabilization by pH 9, and P420 predominant at pH 6. Within the pH range 6.1–10.5, the CYP121 FeII-CO complex could be reversibly titrated between P450 and P420 forms. However, in the ranges above pH 10.5 and below pH 6.0 the protein was denatured irreversibly, with either apparent heme dissociation or protein aggregation and precipitation. Fig. 6 shows plots of data from the pH titration of the CYP121 FeII-CO complex at both 450 and 420 nm. The data are fitted accurately to a single $pK_a$ equation, giving midpoint ($pK_a$) values of 7.2 ± 0.1 for data at both wavelengths, consistent with the protonation of a single residue (i.e. Cys345, the heme iron proximal ligand) underlying the observed P450/P420 optical transition.

In the absence of CO, the complete reduction of CYP121 to its ferrous state is difficult due to the negative potential of the heme iron, and the protein is also prone to aggregation with extended incubation in the presence of the large excess of dithionite required to achieve substantial reduction of the heme iron. However, it is clear from previous redox potentiometry studies and from the optical spectra presented here (Fig. 1B) that reduction of CYP121 from FeIII to FeII is accompanied by a spectral shift from 416.5 to 405 nm, consistent with the maintenance of the proximal ligand in the thiolate form in the reduced, CO-free enzyme. Thus, it appears that coordination of CO as the 6th ligand to the CYP121 heme iron is the major determinant affecting protonation of the proximal cysteinate ligand and the consequent development of the P420 form.

The situation is different for CYP51B1, where our previous redox potentiometry studies have shown that the ferrous, CO-free form (readily formed using dithionite as reductant) has optical properties similar to that of a thiols-coordinated form of myoglobin (18). Thus, cysteine thiolate protonation in CYP51B1 appears to occur at the reduction stage. We examined the spectral features of both the FeIII and FeII-CO forms of CYP51B1 in equilibrium and across a range of pH values. Unlike with CYP121, there was no major interconversion of the P420 and P450 FeII-CO complexes, with the P420 form predominant across the pH range from 6.5 to 10.5.

**Laser Photoexcitation of CYP121 and CYP51B1 in the Absence of CO**—To determine apparent reduction rates for enzymes in the absence of CO, we repeated laser photoexcita-
P450 Heme Iron Reduction of CYP51B1 and CYP121

A

B

FIGURE 6. Determination of midpoint pH for the CYP121 P450/P420 transition. A shows a plot of 450-nm absorption data for the CYP121 (5.6 μM) FeIII-CO complex, collected across the pH range from 6.5 to 10.5. B shows a similar plot of the data at 420 nm. Full equilibration was ensured at each point in the titration prior to spectral acquisition. The titration was done as described under “Experimental Procedures.” Data were fitted to a single pKₐ function, producing apparent pKₐ values of 7.2 ± 0.1 for both data sets.

tion studies with both CYP51B1 and CYP121 in the absence of CO ligand, and with NADPH at 300 μM. Reaction transients were collected at different wavelengths, using spectral data presented in Fig. 1, A and B, to guide the choice of wavelengths at which major changes in the absorption spectrum occur for CYP121 and CYP51B1. For CYP121, transient absorption changes were monoexponential, and reduction of FeIII to FeII heme iron occurred at 10,200 ± 400 s⁻¹ as measured at 400, 416, and 420 nm. The directions of absorption changes at these wavelengths are consistent with the retention of thiolate coordination in the ferrous CYP121 enzyme, which has its absorption maximum at 410 nm. For CYP121, the comparable rates determined were 11,200 ± 350 s⁻¹, as measured at 410, 419, and 423 nm. Again, transient absorption change data were monophasic for CYP51B1. Absorption changes in the same time domain were also observed at 559 nm (at which point there is increased absorption for the FeII form of CYP51B1 at equilibrium, Fig. 1A). However, the magnitude of absorption changes in the visible region was too small to enable accurate fitting of data to an exponential function. Data were also collected at 340 nm to report on laser-mediated oxidation of NAD(P)H under the same experimental conditions. Rates of ~8 × 10⁶ s⁻¹ were obtained, consistent with previous data (21).

Photodiode array analysis of spectral changes (for periods of up to 8 min) following laser photoexcitation of CO-free CYP51B1 demonstrated that a much slower phase of absorption change occurred for the FeIII enzyme, leading eventually to the development of a spectral form as shown in Fig. 1A. The rate of this slower process is ~0.08 min⁻¹ from data analyzed at 423 nm. Thus, we conclude that thiolate coordination of CYP51B1 heme iron is retained immediately following laser-mediated reduction of the P450, but that protonation of the thiolate then occurs in the slow phase to produce the distinctive spectral signature of the FeII form of CYP51B1 (Fig. 1A). Thus, these results are consistent with previous data that indicated that it is the FeII form of CYP121 per se (as opposed to the FeII-CO complex) that is unstable and prone to protonation of the cysteine thiolate (16). The optical signature change associated with thiolate protonation in the FeII-CO complex (i.e. P450 to P420) is distinctive and can be conveniently assayed. However, the binding of CO appears to have little influence on the stability of the thiolate ligand to protonation in the CYP51B1 enzyme, and the cysteinate is readily protonated in the absence of the CO ligand.

The data for heme iron reduction mediated by NAD(P)H photoexcitation in CYP121 and CYP51B1 demonstrate that electron transfer to the P450s (presumably mediated by aqueous electrons released from the coenzyme on laser irradiation) occurs in the absence of CO ligand, and that heme iron reduction is substantially faster than the subsequent formation of the FeII-CO complex. The rates determined for reduction of these enzymes to their FeII forms are comparable with that determined recently for the reduction of the heme domain of flavocytochrome P450 BM3 (~14,000 s⁻¹ under similar reaction conditions) (21).

DISCUSSION

The data presented in this study demonstrate the applicability of laser photoexcitation of pyridine nucleotide coenzymes (NADPH and NADH) for the reduction of the heme iron in two M. tuberculosis P450s (CYP121 and CYP51B1) on a microsecond time scale. No reductive reaction with either P450 or coenzyme is observed in the absence of laser photoexcitation. The technique affords substantial enzyme reduction by a single laser flash (up to ~20% P450 heme iron reduction is typical), and thus provides quite large absorption changes and good signal-to-noise ratios. Moreover, the data provide important new information on the formation, stability, and interconversion of the thiolate-(P450) and thiol-coordinated heme iron forms of these P450s (18).

We have exploited this novel NAD(P)H laser photoexcitation technology to analyze the kinetics of formation of the FeII and FeII-CO complexes of CYP51B1 and CYP121, and to obtain data on affinity for the CO ligand in both cases. The data revealed very distinctive features of these P450s with respect to their CO-binding properties. CYP121 showed a hyperbolic dependence of the rate of FeII-CO complex formation on the concentration of CO, as was also observed for the P450 BM3 heme domain (21). A fit of the CYP121 data to a hyperbolic
function yields an apparent $K_d$ for CO of 914 $\pm$ 139 $\mu$M with NADPH as donor. We consider that this phenomenon is likely due to the presence of a relatively low affinity internal binding site (or sites) for CO in the protein matrix of the Fe(II) enzyme, with relocation of CO to the ferrous iron (forming Fe(III)-CO) occurring on heme iron reduction (Fig. 7). The same model was invoked to explain a similar type of CO dependence in the P450 BM3 enzyme (21). Thus, it is likely that the $K_d$ value determined for CO reports on the affinity of CO for the ferric CYP121 protein. The reaction transients obtained for the CYP121 Fe(II)-CO complex formation were consistently biphasic, which could be explained by conformational heterogeneity in the enzyme sample, or possibly distinct binding sites for CO in the ferric enzyme. Structural studies on CYP121 reveal a single conformation with a constricted active site (12, 13). However, catalysis should involve conformational change to facilitate substrate access to the heme iron, and thus it may be expected that different conformations of the P450 are also represented in the solution state. An alternative explanation for biphasic reaction kinetics could be the involvement of two different reducing species following laser photoexcitation of NAD(P)H. Whereas we invoke aqueous electrons released from coenzyme as the major reducing species, it is also plausible that the NAD(P)$^+$ radical acts as a reductant (Fig. 7) (27, 28). This species is formed by rapid deprotonation of NAD(P)(H)$^+$, which (along with the aqueous electron) is the product of laser photolysis of NAD(P)H. However, the monophasic reduction kinetics for the CO-free forms of both CYP121 and CYP51B1, and the monophasic transients associated with CYP51B1 Fe(II)-CO complex formation suggest that conformational equilibria or distinct CO binding sites may be more likely explanations for the biphasic kinetics observed in CYP121.

For the studies of CYP121 Fe(II)-CO complex formation (Fig. 2B), it is clear that NADH and NADPH are equally effective reductants, indicating that the presence of the 2' phosphate group on the latter makes negligible difference to its relative capacity to act as reductant in these experiments. Some differences in the reductive kinetics and apparent $K_d$ for CO were observed between NADH/NADPH in a previous photoexcitation study of the P450 BM3 heme domain (21). A possible explanation for the BM3 data is allosteric effects mediated by one or both coenzymes. Given the absence of significant differences between NADPH/NADH in the CYP121 system, such effects can be ruled out for this P450.

Importantly, photoexcitation data at various pH values demonstrated that the Fe(III)-CO complex initially formed by CYP121 is that of a thiolate-coordinated enzyme, i.e. P450 (with Soret maximum at 448 nm). Only after formation of the P450 complex does thiolate protonation occur and P420 develop (with Soret maximum at 420 nm) (Fig. 7). The extent of formation of P420 is pH-dependent, with more extensive P420 accumulation at lower pH (Fig. 3B). Equilibrium pH titration of the Fe(II)-CO complex reveals a pH-dependent equilibrium of P450/P420 that is completely reversible within the pH range 6.5–10.5, and with an apparent $pK_a$ value of 7.2. To our knowledge, this is the first demonstration of such a P450/P420 equilibrium for a P450 enzyme, and provides strong evidence for the role of cysteine thiolate protonation in the conversion from P450 to P420. It is also of interest to note that the CYP121 P450/P420 equilibrium can be perturbed by varying temperature (data not shown), with a greater amount of the P450 Fe(II)-CO complex formed at lower temperatures.

Whereas it is the Fe(II)-CO species that is readily protonated/deprotonated to form P420/P450 in CYP121, it is the Fe(III) species in CYP51B1 that is unstable and readily undergoes thiolate protonation. Another major variation in CYP51B1 behavior (by comparison with CYP121) is evident in the kinetic transients accompanying Fe(III)-CO complex formation. For CYP51B1 these are monophasic, and the plot of the observed Fe(II)-CO complex formation rate versus CO concentration is linear rather than hyperbolic. This second order dependence suggests
the absence of defined site(s) for CO in the ferric CYP51B1 enzyme (Fig. 7).

For both CYP51B1 and CYP121, reduction of ferric heme iron occurs much faster (~10,000–11,000 s\(^{-1}\)) than does Fe\(^{II}\)-CO complex formation. For CYP121 the limiting rate constant for Fe\(^{II}\)-CO complex formation \((k_{lim})\) is ~1,900 s\(^{-1}\), whereas for CYP51B1 the second order rate constant is 0.185 \(\mu\)M\(^{-1}\) s\(^{-1}\) (with NADPH as electron donor in both cases). Thus, the rate for CYP51B1 at saturating CO (~975 \(\mu\)M) is ~180 s\(^{-1}\). The lower rate for CYP51B1 might also be consistent with the absence of a low affinity CO-binding site in the ferric enzyme, and thus a slower progression of CO to the Fe\(^{II}\) CYP51B1 heme iron. On binding estradiol, the second order binding rate constant for CO is decreased (to 0.134 \(\mu\)M\(^{-1}\) s\(^{-1}\)), consistent with steric hindrance to the CO ligation site.

In conclusion, we present data demonstrating the exploitation of laser excitation of NAD(P)H coenzymes as a route to rapid reduction of the ferric heme iron in two structurally characterized \(M. \) tuberculous P450 enzymes. These, and accompanying equilibrium binding data, demonstrate that heme iron reduction in both CYP121 and CYP51B1 is rapid with respect to the CO binding that leads to formation of the characteristic Fe\(^{II}\)-CO (P450) complex. However, the data also show that the ferric enzymes in both cases are thiolate-coordinated (as confirmed by EPR studies), and that heme iron reduction in both cases results in formation of the thiolate-coordinated Fe\(^{II}\) enzyme (11, 16, 26, 29). In the case of CYP121 (and in absence of CO), this Fe\(^{II}\) species collapses to the thiol-coordinated form observed at equilibrium, and at a rate of ~0.08 min\(^{-1}\). In the case of CYP121, the thiolate-coordinated Fe\(^{II}\) form is relatively stable and predominant at equilibrium. However, the Fe\(^{II}\)-CO complex is unstable in the thiolate-coordinated state (unless at basic pH) and is readily protonated to the thiol-coordinated P420 species under acidic conditions. The rate for the P450 to P420 conversion in CYP121 is ~0.1 s\(^{-1}\) at pH 7.0. These data demonstrate the applicability of the NAD(P)H photoexcitation method as a route to rapid reduction of P450 heme, with obvious ramifications for isolation of transient oxy complexes relevant to catalysis. However, they also highlight important differences between two P450 isoforms in terms of their interactions with CO and the relative propensity of their Fe\(^{II}\) or Fe\(^{II}\)-CO species to undergo protonation of heme cysteinate and form the thiol-coordinated P420 state. They also provide clear evidence for a reversible pH-dependent P450/P420 equilibrium in the CYP121 Fe\(^{II}\)-CO complex, dispelling dated notions that the P420 species marks a structurally disrupted and irreversibly inactivated form of P450. It is patent from this work that a reversible protonation in CYP121 underlies the P450/P420 optical transition.

**REFERENCES**

1. World Health Organization (2007) Fact Sheet Number 104
2. Cole, S. T., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., Gordon, S. V., Ewigmeier, K., Gas, S., Barry, C. E., III, Tekeia, F., Badcock, K., Basham, D., Brown, D., Chillingworth, T., Connor, R., Davies, R., Devlin, K., Feltwell, T., Gentsles, S., Hamlin, N., Holroyd, S., Hornsby, T., Jagels, K., Krogh, A., McLean, J., Moule, S., Murphy, L., Oliver, K., Osborne, I., Quail, M. A., Rajandream, M. A., Rogers, J., Rutter, S., Seeger, K., Skelton, I., Squares, R., Squares, S., Sulston, J. E., Taylor, K., Whitehead, S., and Barrell, B. G. (1998) *Nature* **393**, 537–544
3. Aoyama, Y., Horiiuchi, T., Gotoh, O., Nisho, M., and Yoshida, Y. (1998) *J. Biochem. (Tokyo)* **124**, 694–696
4. Bellamine, A., Mangla, A. T., Nes, W. D., and Waterman, M. R. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 8937–8942
5. Waterman, M. R., and Lepesheva, G. I. (2005) *Biochem. Biophys. Res. Comm.* **338**, 418–422
6. Lepesheva, G. I., and Waterman, M. R. (2004) *Mol. Cell. Endocrinol.* **215**, 165–170
7. McLean, K. J., Marshall, K. R., Richmond, A., Hunter, I. S., Fowler, K., Kieser, T., Gurcha, S. S., Besra, G. S., and Munro, A. W. (2002) *Microbiology* **148**, 2937–2949
8. Guardiola-Diaz, H. M., Foster, L. A., Mushrush, D., and Vaz, A. D. (2001) *Biochem. Pharmacol.* **61**, 1463–1470
9. Jackson, C. J., Lamb, D. C., Kelly, D. E., and Kelly, S. L. (2000) *FEBS Microbiol. Lett.* **192**, 159–162
10. Ahmad, Z., Sharma, S., and Khuller, G. K. (2006) *FEBS Microbiol. Lett.* **261**, 181–186
11. McLean, K. J., Cheesman, M. R., Rivers, S. L., Richmond, A., Leys, D., Chapman, S. K., Reid, G. A., Price, N. C., Kelly, S. M., Clarkson, J., Smith, W. E., and Munro, A. W. (2002) *J. Inorg. Biochem.* **91**, 527–541
12. Leys, D., Mowat, C. G., McLean, K. J., Richmond, A., Chapman, S. K., Wankinshaw, M. D., and Munro, A. W. (2003) *J. Biol. Chem.* **278**, 5141–5147
13. Seward, H. E., Roujeinikova, A., McLean, K. J., Munro, A. W., and Leys, D. (2006) *J. Biol. Chem.* **281**, 39437–39443
14. Podust, L. M., Poulos, T. L., and Waterman, M. R. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98**, 3068–3073
15. Podust, L. M., Vermanis, L. P., Lepesheva, G. I., Podust, L. M., Dalmaso, E. A., and Waterman, M. R. (2004) *Structure* **12**, 1937–1945
16. McLean, K. J., Warman, A. J., Seward, H. E., Marshall, K. R., Girvan, H. M., Cheesman, M. R., Waterman, M. R., and Munro, A. W. (2006) *Biochemistry* **45**, 8427–8443
17. Estabrook, R. W. (2003) *Drug Metab. Dispos.* **31**, 1461–1473
18. Perera, R., Sono, M., Sigman, J. A., Pfister, T. D., Lu, Y., and Dawson, J. H. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100**, 3641–3646
19. Munro, A. W., Lindsay, J. G., Coggins, J. R., Kelly, S. M., and Price, N. C. (1996) *Biochim. Biophys. Acta* **1296**, 127–137
20. Ogura, H., Nishida, C. R., Hoch, U. R., Perera, R., Dawson, J. H., and Ortiz de Montellano, P. R. (2004) *Biochemistry* **43**, 14712–14721
21. Girvan, H. M., Heyes, D. J., Scrutton, N. S., and Munro, A. W. (2007) *J. Am. Chem. Soc.* **129**, 6447–6453
22. Cargill, R. (ed) (1990) *Carbon Monoxide*, pp. 5–30, Vol. 43, Pergamon Press, Oxford
23. Omura, T., and Sato, R. (1964) *J. Biol. Chem.* **239**, 2370–2378
24. Miles, J. S., Munro, A. W., Rospodowski, B. N., Smith, W. E., McKnight, J. E., and Thomson, A. J. (1992) *Biochem. J.* **288**, 503–509
25. Sligar, S. G., and Gunsalus, I. C. (1976) *Biochemistry* **14**, 2997–3001
26. Daff, S. N., Chapman, S. K., Turner, K. L., Holt, R. A., Govindaraj, S., Poulos, T. L., and Munro, A. W. (1997) *Biochemistry* **36**, 13816–13821
27. Ortiz, Y. (1993) *Biochemistry* **32**, 11910–11914
28. Czochralska, B., and Lindqvist, L. (1983) *Chem. Phys. Lett.* **101**, 297–299
29. Matsuura, K., Yoshioka, S., Tosha, T., Hori, H., Ishimori, K., Kitagawa, T., Morishima, I., Kagawa, N., and Waterman, M. R. (2005) *J. Biol. Chem.* **280**, 9088–9096