

12-1-2013

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This is the peer reviewed version of the article appearing in *Journal of Raman Spectroscopy*, Vol. 44, No. 12 (December 2013): 1792–1794. It has been published in final form here: [DOI](#). © 2013 Wiley. This article may be used for non-commercial purposes in accordance With Wiley Terms and Conditions for self-archiving.

Using Resonance Raman Cross-section Data to Estimate the Spin State Populations of Cytochromes P450

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Abstract:

The cytochromes P450 (CYPs) are heme proteins responsible for the oxidation of xenobiotics and pharmaceuticals and the biosynthesis of essential steroid products. In all cases, substrate binding initiates the enzymatic cycle, converting ferric low spin (LS) to high-spin (HS), with the efficiency of the conversion varying widely for different substrates, so documentation of this conversion for a given substrate is an important objective. Resonance Raman (rR) spectroscopy can effectively yield distinctive frequencies for the ν_3 "spin state marker" bands. Here, employing a reference cytochrome P450

(CYP101), the intensities of the ν_3 modes (I_{LS}) and (I_{HS}) relative to an internal standard (sodium sulfate) yield relative populations for the two spin states; i.e., a value of 1.24 was determined for the ratio of the relative cross sections for the ν_3 modes. Use of this value was then shown to permit a reliable calculation of relative populations of the two spin states from rR spectra of several other Cytochromes P450. The importance of this work is that, using this information, it is now possible to conveniently document by rR the spin state population without conducting separate experiments requiring different analytical methods, instrumentation and additional sample.

Keywords: Cytochrome P450, Raman, spin state.

The cytochromes P450 (CYPs) are heme-based monooxygenases responsible for the oxidative metabolism of a huge number of relatively inert substrates, including pharmaceuticals and other xenobiotics, and for the biosynthesis of essential steroid products.^[1-4] Substrate binding initiates the enzymatic cycle by triggering a crucial low spin (LS) to high spin (HS) state change that facilitates reduction to the ferrous heme intermediate that rapidly binds oxygen, which is then converted to the highly reactive Compound I.^[5] Inasmuch as the efficiency of spin state conversion varies widely for different substrates and enzymes,^[1,2,6,7] documentation of the extent of this conversion for a given substrate is an important experimental objective. Resonance Raman (rR) spectroscopy can effectively document the *presence* of HS or LS states of the ferric heme by appearance of distinctive frequencies observed at ~ 1485 and 1500 cm^{-1} , respectively.^[8-10] Given recent advances in producing and stabilizing these extremely important enzymes,^[11-13] it is anticipated that applications of rR to these proteins will now expand considerably. The purpose of this work is to provide a systematic approach to utilize rR spectroscopy to reliably estimate spin state populations for different substrate/enzyme combinations.

Cytochrome P450cam (CYP101), an ideal reference protein, exhibits an almost complete spin state conversion upon binding its natural substrate, camphor, switching from 96% LS to 95% HS, as documented by electronic absorption spectrophotometry, Fig. S1 (Supporting Information).^[14] The CYP101 was expressed and purified as published earlier;^[11,15] experimental procedures, including sample preparation for rR measurements, are presented in Supporting

Information. The laser excitation lines for these studies were 406.7 and 413.1 nm.^[8-10, 16-19]

As shown in Figure 1, the spin state change is observed as a shift of ν_3 from 1502 cm^{-1} (LS) to 1488 cm^{-1} (HS). To estimate relative rR cross sections for the substrate-free (SF) and substrate-bound (SB) samples, three samples of these two forms, each containing 0.500 M internal standard Na_2SO_4 , were measured and their spectra were analyzed using the following peak fitting procedure. The SO_4^{2-} bands at 981 cm^{-1} (and all heme modes) were found to fit best with a 30% Gaussian/ 70% Lorentzian function. The average band width for the sulfate mode for all six spectra was 10.1 cm^{-1} ; the resulting band widths of the spin-marker bands were 11.5 cm^{-1} (1488 cm^{-1}) and 11.3 cm^{-1} (1502 cm^{-1}). Peak areas, instead of peak heights, were used to calculate the relative cross sections of the spin state marker bands; i.e., $I_{\text{LS}}/I_{\text{IS}}$ and $I_{\text{HS}}/I_{\text{IS}}$, where I_{IS} is the intensity of the 981 cm^{-1} band of sulfate. Noting that SF CYP101 is 96 % low spin and the camphor-bound is 95% high spin,^[14] the operative relative intensities, Y_{LS} and Y_{HS} , values were derived by dividing the raw relative intensities by 0.96 and 0.95 factors, respectively. The $Y_{\text{HS}}/Y_{\text{LS}}$ ratios of the ν_3 bands were calculated for all nine combinations derived from six samples and the results are presented in Table S1 of Supporting Information. The $Y_{\text{HS}}/Y_{\text{LS}}$ ratio for the ν_3 mode with 406.7 nm excitation line is 1.24 ± 0.06 ; similar calculations for spectra measured with the 413.1 nm excitation line yielded a $Y_{\text{HS}}/Y_{\text{LS}}$ ratio of 1.19 ± 0.04 . Though of borderline significance, this smaller value is reasonable, because the 413.1 nm line is closer to resonance with the Soret band of the LS sample (417 nm) (Table S1, Supporting Information).

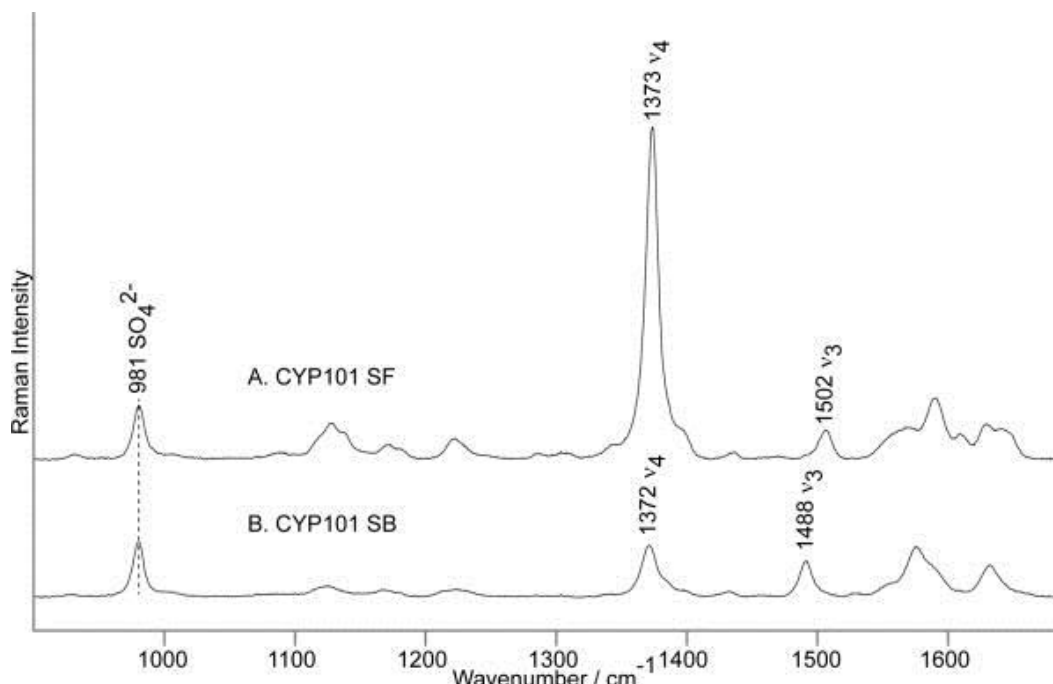


Figure 1 The resonance Raman spectra of ferric CYP101 substrate-free (A) and substrate-bound (B). Spectra measured with 406.7 nm excitation line and normalized to the sulfate band at 981 cm⁻¹.

In order to expand potential applications of this procedure, these ratios were also calculated for the ν₄ and ν₇ modes with both excitation lines (Table S2, Supporting Information). These data can be used to normalize spectra in different regions. One can apply the Y_{HS}/Y_{LS} ratio of 0.21 ± 0.013 for the intense ν₄ mode when normalizing high frequency spectra (Fig. S2, Supporting Information) and in the low frequency region one could utilize the 0.38 ± 0.020 ratio for ν₇ mode.

Given that the electronic spectra of both the HS and LS states of the bacterial CYPs correspond well with those of mammalian CYPs,^[20] it is reasonable to expect that the value of 1.24 derived here for CYP101 should be valid for spectra of mammalian CYPs. To evaluate this issue, the derived 1.24 value was applied to calculate the percentage of spin state conversion upon substrate binding of several mammalian cytochromes available in our laboratory; i.e., CYP2B4, ND:CYP3A4 and ND:CYP17.^[21-25] The percentages of LS and HS states calculated from rR spectra (406.7 nm excitation) using the method presented above were compared with percentages independently derived from available UV-Vis data (Table 1). As can be seen, the data match quite well for CYP2B4 with butylated hydroxytoluene (BHT),

CYP3A4 both SF and with erythromycin (ERY) as well as CYP17 with progesterone (PROG) and 17-hydroxyprogesterone (17-OH PROG). The value obtained here for the testosterone (TST)-bound ND:CYP3A4 falls within the range reported for measurements with UV-visible spectrophotometry; as is discussed more thoroughly in several earlier works dealing with substrate-binding equilibria and spin state conversion for these systems,^[22-24] the percentage LS→ HS conversion is not linearly related to the number of bound substrates and is further complicated by the fact that substrate access to enzyme also depends on partitioning of the substrate into the lipid bilayer of the nanodisc. Similar complications can also be encountered for the bromocryptine (BC)-bound samples of the ND:CYP3A4 system and may account for the slightly larger, but not unreasonable, discrepancy seen here; i.e., 80% vs 93%.^[26]

protein	Resonance Raman		UV-Vis	
	% HS	% LS	% HS	% LS
CYP2B4				
+ BHT	94	6	93 ^a	7 ^a
CYP3A4 SF	5	95	11 ^b	89 ^b
+ TST	74	26	80-92 ^{b,c}	8 ^{b,c}
+ BC	80	20	93 ^b	7 ^b
+ ERY	19	81	22 ^b	78 ^b
CYP17				
+ PROG	94	6	97 ^d	3 ^d
+ 17-OH PROG	59	41	62 ^d	38 ^d

Table 1 The calculated percentage of spin state populations in various cytochromes P450 measured with 406.7 nm excitation line and using the Y_{HS}/Y_{LS} ratio of 1.24 the ν_3 modes. The calculated data are compared with the data derived from UV-Vis spectra.

^areference ²¹.

^breference ²²

^creference ²³

^dreference ²⁵.

Supplementary Material

Supporting Information

Using resonance Raman cross-section data to estimate the spin state populations of Cytochromes P450

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Acknowledgments

This work was supported in part by grants from the National Institutes of Health (GM96117) and the National Science Foundation (MCB 0951110) to JRK.

Footnotes

Supporting information

Supporting information may be found in the online version of this article.

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