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Temperature Derivative Spectroscopy to monitor the autoxidation decay of cytochromes P450

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Abstract

Temperature Derivative Spectroscopy, a type of relaxation spectroscopy, is a powerful tool to study protein dynamics¹. We developed the version of Temperature Derivative Spectroscopy (TDS) to monitor kinetics of autoxidation of cytochromes P450 and applied it to study the properties of the oxy-ferrous complex of a human membrane bound P450, CYP19A1 (aromatase), and that of a bacterial soluble P450, CYP101 when bound with their most common substrates, androstenedione (AD) and camphor respectively. TDS extends the panel of methods that can be used to monitor heme protein kinetics, providing a rapid measurement technique and enabling measurement of autoxidation rate over a wide range of temperatures yielding the activation energy as well as absolute reaction rate in a single experiment.

Keywords

Temperature Derivative Spectroscopy; Cytochrome P450; Oxy-ferrous heme complex; Autoxidation

INTRODUCTION

Temperature Derivative Spectroscopy¹ (TDS) is a type of relaxation spectroscopy that involves a temperature dependent ‘perturbation’ and a corresponding ‘observable’. The perturbation could be formation or decay of a molecular species by photolysis, ligand binding or a mere change in temperature. The list of ‘observables’ that could be used with TDS includes Raman scattering, circular dichroism, fluorescence anisotropy and UV-Vis absorption spectroscopy.. Previously, TDS has been used extensively to study various aspects of ligand binding and dynamics in myoglobin^{2,3} and neuroglobin⁴. We have developed temperature derivative spectroscopy as a means to measure the kinetics of autoxidation decay of the oxy-ferrous complex in cytochrome P450 and applied it to the two members of the cytochrome P450 superfamily, CYP19A1 (human aromatase) and CYP101 (P450cam).

The cytochromes P450 constitute a large family of thiolate-ligated heme proteins present in plants, bacteria and mammals that play an important role in the oxidative transformation of various endogenous and exogenous molecules⁵. This superfamily of enzymes catalyzes a variety of reactions starting from simple hydroxylations as in CYP101⁶, the most well

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studied bacterial P450, to N-demethylation and O-dealkylation in CYP3A4⁷, a hepatic P450 that is involved in xenobiotic metabolism in mammals.

Each catalytic cycle of P450s (Figure 1) requires a molecule of O₂ and two reducing equivalents, usually originating from NADPH through action of an associated electron transfer protein. The P450 reaction cycle contains three branch points where side reactions occur. Together, referred to as uncoupling pathways, these include in order of occurrence an autoxidation of the ferrous oxy complex, release of peroxide following second electron transfer and an oxidase shunt where a second molecule of water is generated (Figure 1).

Autoxidation constitutes the first unproductive pathway in P450 catalysis wherein release of a superoxide anion from the oxy-ferrous complex in P450s returns the enzyme to its ferric resting state⁸. The autoxidation shunt constitutes the major unproductive pathway for many P450s⁹ and thus its quantitation and mechanism of action is of interest to pharmacologists, toxicologists and biochemists. Autoxidation is intimately coupled to the redox potential of heme iron in these enzyme systems¹⁰ and different substrates exercise their control on the rates of autoxidation primarily by modulating the mid-point reduction potential of the heme iron.

The rates of autoxidation in P450s range from 0.005 s⁻¹ in camphor bound-CYP101¹¹ to 20 s⁻¹ in substrate-free CYP3A4 at 5 °C⁹. It is known that the autoxidation shunt accounts for a major fraction of reactive oxygen species (ROS) in mammalian P450 CYP11A1, both *in vivo* and *in vitro*¹² and this can lead to the induction of apoptosis in cells.

The oxy-ferrous complexes of various cytochromes P450 have been spectroscopically characterized¹³. However, the more physiologically relevant information is the autoxidation rates and the energy of activation for the conversion of the oxy-ferrous complex into the ferric resting state of the enzyme. The most commonly used method to investigate the autoxidation of cytochromes P450 in the 0.01 – 10 second time range is stopped-flow spectroscopy. However, this requires special equipment and substantial amounts of enzyme which normally cannot be recovered after experiments. Alternatively, TDS provides a relatively easy and quick method to obtain the same information using a regular slow scanning spectrophotometer and an easily assembled optical cryostat. Because the measurements are done at lower temperatures, where all reaction rates are dramatically slowed, TDS makes possible studies of fast reactions without access to expensive instrumentation. The Nanodisc technology allows the same method to be used with the membrane proteins solubilized in the native bilayer environment at a known oligomerization state¹⁴. In monitoring autoxidation using TDS, the ‘perturbation’ and the ‘observable’ are the decay of oxy-P450 and the spectral changes in the UV-Vis region induced by conversion of the oxy-ferrous complex to the ferric form.

CYP101, or P450cam, is a soluble cytochrome P450 from *Pseudomonas putida* that was available in large quantities and was instrumental in the exploration of the mechanistic complexities of this enzyme superfamily¹⁵. CYP19A1 is a membrane-bound P450, localized in the endoplasmic reticulum of the cell, whose function in humans is to convert androgens to estrogens. It forms an important part of the steroidogenesis pathway in humans and is the target for frontline therapy of estrogen receptor positive breast cancer in post-menopausal women¹⁶. The oxy-ferrous complexes of both these enzymes in their substrate bound forms have been previously observed^{11,17} and therefore provide good reference points for these studies using TDS to characterize the kinetics and activation parameters for oxy-ferrous complex decay.

MATERIALS AND METHODS

Expression and purification of CYP101

Expression of CYP101 in *E. coli* and protein purification were performed as described¹⁸. Mutagenesis of the CYP101 gene (pCamT7) was performed using the Stratagene Quikchange kit following the manufacturer's recommendations. The CYP101 gene with a hexahistidine tag added at the C-terminal (pT7-P450 His) was expressed in BL21 (DE3) cells (Stratagene) and grown overnight in the presence of 0.5 mM δ -aminolevulinic acid. After cell sonication, resuspension in 50 mM KPi pH 7.4 containing 150 mM KCl (buffer A), and ultracentrifugation the protein extract was loaded on a Ni-NTA column (Amersham), washed with buffer A in the presence of 50 mM imidazole, and eluted with the same buffer supplemented with 20 mM EDTA. The resulting red fractions were dialyzed in 50 mM KPi, 400 μ M D-camphor, 20 mM β -mercaptoethanol, pH 7.4 (designated as RB buffer) overnight at 4 °C. Solid ammonium sulfate was added to 30% saturation over 30 minutes and the protein was subsequently loaded onto a phenyl-sepharose CL6B column equilibrated in the same buffer and eluted in a linear gradient from 30% to 0% ammonium sulfate. Resulting fractions with an R_z-value (A_{392}/A_{280}) greater than 1.45 were judged as pure and concentrated using an Amicon cell.

Expression, purification and self-assembly of CYP19A1 in Nanodiscs

CYP19A1 was expressed and purified in a detergent solubilized form as previously described¹⁹. CYP19A1 is a membrane bound enzyme. In order to obtain a monodisperse, homogenous and a stable enzyme preparation it was incorporated into Nanodiscs. Nanodiscs provide a native-like membrane environment for the protein where it maintains its full functionality over a wide range of experimental conditions, and enable it to withstand the full rigor of various biophysical tools¹⁴. Nanodisc technology uses an engineered amphipathic protein, referred to as the Membrane Scaffold Protein (MSP) that interacts with phospholipids on one side and aqueous solvent on the other side to form discoidal lipid bilayers in which integral membrane proteins assemble^{20,21}. The procedure for the self-assembly of CYP19A1 in Nanodiscs has been described in detail¹⁹.

Preparation of the ferrous oxy-complexes of P450

A 60 μ l of a mixture of 100 μ M CYP19A1 in palmitoyl-oleoyl-phosphatidyl-choline (POPC) Nanodiscs, 125 μ M 4-Androstenedione (obtained from Sigma) and 2.5 μ M methylviologen in 100 mM phosphate buffer, pH 7.4 was taken in a small glass vial with a PTFE/Red rubber septa (SUPELCO) and deoxygenated by Argon, under rotation, for 3–4 minutes. Separately, 540 μ l of a solution containing 75 % glycerol, 125 μ M substrate and 100 mM phosphate buffer, pH 7.4 was prepared in a methacrylate cuvette (4.3 mm path length UV-enhanced semimicro cells, Fisher) and saturated using pure oxygen by bubbling and occasional stirring for 15 minutes.

Concentrated CYP19A1 with 125 μ M AD, methylviologen (MV: CYP19A1 molar ratio of 1:50), 100 mM KPi, pH 7.4, and 15% glycerol was deoxygenated and reduced with 2.5 M equivalents of dithionite in a Coy Labs anaerobic glove box. The dithionite concentration was determined using its molar absorption coefficient of 8.05 mM⁻¹ cm⁻¹ at 315 nm²². To form the oxy-ferrous complex the reduced CYP19A1 + AD sample in glycerol was manually injected into a solution of aerobic glycerol-containing buffer precooled to 243 K, mixed for 45 s, transferred to a 200 K dry ice/ethanol bath and stored in liquid nitrogen before further use.

The same procedure was used to form the oxy-complex of soluble CYP101, except no methylviologen was used.

Thermal annealing instrumentation

The experiments were performed in a home-built cryostat setup previously described in ²³. The cryostat comprises an unsilvered Pyrex glass dewar that is transparent to wavelengths above 300 nm. A holder attached to the top cover of the dewar houses the cuvette when the experiment is on. Prior to beginning the experiment, the dewar is precooled to 200 K using liquid nitrogen at the bottom of the dewar, added through a funnel attached to the top cover of the dewar. The sample is never in direct contact with liquid nitrogen to prevent cracks and maintain the optical transparency. A thermocouple in contact with the sample solution was used to monitor the temperature of the sample during measurement.

Choosing an optimal temperature ramping rate

Figure 2 contains a series of calculated curves depicting the decay of oxy-ferrous complex, generated using equation 1 with an 18 kcal/mol Arrhenius activation energy ¹⁷, and different ramp rates. This graph illustrates how the ramping rate influences the time-span of an experiment and therefore, the number of data points available for analysis. The number of available data points is determined by the ability of the instrumentation to record high quality spectra at the rate of data acquisition which defines the level of accuracy in extracting parameters such as E_a (activation energy) and k_T (rate of autoxidation at a particular temperature). For instance, at the scanning rate 600 nm/min, one can measure the full spectrum in the range 750 – 300 nm in 1 minute, which corresponds to one spectrum per degree Kelvin at a temperature ramping rate 1 K/s.

For a first-order reaction involving conversion of a species S to product P the rate of disappearance of S can be calculated using the Arrhenius equation and represented mathematically as:

$$\frac{dS}{dt} = - \left(A \exp \left(\frac{-E_a}{RT} \right) \right) \cdot S$$

Rearranging we get,

$$\frac{dS}{S} = - A \exp \left(\frac{-E_a}{RT} \right) \cdot dt$$

For a linear temperature-time relationship, $dT = m \cdot dt$. Here, m is the slope of the temperature-time profile. Substituting for dt and integrating within proper limits we get,

$$\int_S^0 \frac{dS}{S} = \frac{-1}{m} \cdot \int_{T_1}^{T_2} A \exp \left(\frac{-E_a}{RT} \right) dT \quad (1)$$

Data acquisition

All UV-Vis absorption spectra were acquired with a Cary 300 Bio UV-Visible Spectrophotometer (Varian Instruments), in a continuous scan mode, at a scan rate of 600 nm/min and a 45 second gap between scans. A thermocouple inserted into the sample cuvette was used to monitor temperature. Temperature readings were noted at the start and end of each UV-Vis scan and averaged for analysis.

Data Analysis

The raw spectral data from the spectrophotometer were first corrected for baseline shifts between scans by subtracting the average absorbance at 595 – 600 nm of each spectrum. The baseline corrected, and time-dependent data was analyzed by a Singular Value Decomposition (SVD) routine in MATLAB to identify the eigenvectors and eigenvalues of the reaction. SVD is a linear-algebra technique that allows one to determine the number of spectrally distinguishable components that are involved in a reaction and to extract maximum information about transition between these species from experimental data set ²⁴.

Figure 3a shows the TDS data for AD-bound-CYP19 reconstructed using the first two eigen vectors. Isosbestic points show a single transition, which is the decomposition of the oxy-ferrous complex into the ferric resting state. The small drift at the isosbestic point at 408 nm is due to the temperature dependent changes of the band shape of both components, i.e. substantial narrowing and sharpening of Soret bands at low temperatures.

Figure 3b shows the first four abstract absorption spectra multiplied by their corresponding singular values for the autoxidation of P450. Only the first two main spectral components are significant, with others being close to zero. This clearly indicates, along with isosbestic points in the raw data, the presence of only two major absorbing species in the data set. Hence, the second concentration vector from SVD is a measure of disappearance of the oxy-ferrous complex or, appearance of the ferric complex. These difference spectra were normalized and fitted to a modified rate equation derived based on the Kramers' theory to account for variable temperature (equation 2). The time and temperature increments were used directly as time steps for integration.

Fitting the concentration profile to the TDS equation

The autoxidation of oxy-CYP101 and oxy-CYP19A1 in their substrate bound states can be modeled as a first-order reaction. For reactions occurring in the high-friction/high-viscosity regime, a modified Kramers' theory ²⁵ provides a good description of the reaction kinetics where the rate constant for a biochemical reaction can be expressed as:

$$k = \frac{A}{\eta^p} \exp(-E_a/RT) \quad (2)$$

Here, A is a frequency (or preexponential) factor, η , the solvent viscosity and E_a , R and T, the Arrhenius activation energy, gas constant and temperature (in Kelvin) respectively ²⁵. p is an empirical exponent smaller than unity, which has been shown to be related to the molecular weight of the cosolvent in question and estimated as 0.54 for glycerol ²⁵.

Samples prepared for TDS contain 70% glycerol and are hence, highly viscous. Since autoxidation involves a dynamic process of opening of the active site to release the superoxide anion, the phenomenon is affected by the viscosity of the solution. It is well known that viscosity of pure liquids and their mixtures depend on temperature and pressure. Very recently, González *et al.* ²⁶ proposed an equation for calculation of the dynamic viscosity of aqueous-glycerol mixtures over a wide range of temperatures and compositions. In fitting the TDS data, a value of 0.65 was used as the weight fraction of glycerol.

Using p as 0.54, and the model for temperature dependence of viscosity proposed by González *et al.* ²⁶ the TDS data for autoxidation decay of the oxy-ferrous complex for camphor-bound CYP101 and AD-bound-CYP19A1 was fitted to the equation 3 in the form given below.

For a first-order reaction involving decay of species S (representing the oxy-complex of cytochrome P450),

$$\begin{aligned}\frac{dS}{dt} &= -k \cdot S \\ k &= \frac{C}{\eta^p} \exp\left(\frac{-E_a}{RT}\right) \\ \Rightarrow \frac{dS}{dt} &= -\frac{C}{\eta^p} \exp\left(\frac{-E_a}{RT}\right) \cdot S\end{aligned}\quad (a)$$

The rate constant 'k' can be alternatively expressed as,

$$k = \frac{C}{\eta^p} \exp\left(\frac{-E_a}{RT}\right) = \left(\frac{k_{298K} \eta_{298K}^p}{\eta^p}\right) \cdot \exp\left(\frac{-E_a}{R} \left(\frac{298 - T}{298T}\right)\right) \quad (b)$$

From (a) and (b),

$$\frac{dS}{S} = -\left(\frac{k_{298K} \eta_{298K}^p}{\eta^p}\right) \cdot \exp\left(\frac{-E_a}{R} \left(\frac{298 - T}{298T}\right)\right) dt$$

Integrating within proper limits for S and T we get,

$$\Rightarrow \int_S^0 \frac{dS}{S} = -\left(\int_{T_1}^{T_2} \left(\frac{k_{298K} \eta_{298K}^p}{\eta^p}\right) \cdot \exp\left(\frac{-E_a}{R} \left(\frac{298 - T}{298T}\right)\right) dT\right) \cdot \frac{dt}{dT} \quad (3)$$

Fitting was accomplished with a program written in MATLAB, using the subroutine 'fminsearch.m' that implements the Nelder-Mead Simplex algorithm to find the minimum of a multiparametric, non-linear function.

RESULTS AND DISCUSSION

As shown in Figure 3a, the starting spectra of oxygenated CYP19A1 in 70% glycerol are almost identical to those previously measured using either stopped flow¹⁷ or cryospectroscopy¹⁹. At 220 – 230 K the rate of autoxidation is very low, and the oxy-complex remains stable. As the temperature increases, the rate of autoxidation also increases, and the oxy-ferrous complex starts to decompose, as shown in Figure 3c. When the temperature reaches 260 K, the rate of autoxidation becomes high enough to change significantly the fraction of oxy-complex during the time of single scan (45 s). The curve shown in Figure 3c reaches zero at 285 K, where all of the oxy-CYP19A1 returns to the high-spin ferric form, as seen from Figure 3a.

The energy of activation for the conversion of oxy-ferrous CYP19A1, saturated with androstenedione, to ferric CYP19A1 was calculated to be 17.5 kcal/mol with a rate constant of 0.02 s⁻¹ at 298 K (Figure 3c). In the case of CYP101, saturated with camphor, this value was determined to be 18.0 kcal/mole with a rate constant of 0.015 s⁻¹ at 298 K (Figure S-4). The values of E_a obtained, in both cases, correspond well with those published earlier^{11,17}. The results obtained using TDS approach with a single sample match almost exactly the parameters derived in earlier stopped-flow studies, which confirms the validity of TDS approach to the autoxidation processes in heme enzymes.

Cytochromes P450s do not possess a static channel for the access of O₂ to the active site. Therefore, the protein has to undergo a conformational change to form a dynamic path for the entry and egress of ligands such as O₂ and CO. The high activation energy barrier for autoxidation (17 kcal/mol for camphor-bound-CYP101¹¹ and 18 kcal/mol in AD-bound-CYP19A1¹⁷) reinforce this idea. An even higher activation energy, 22 kcal/mol was reported for the autoxidation of CYP11A1, another P450 involved in steroid hormone biosynthesis²⁷, although this measurement was made in the presence of 38% ethylene glycol at subzero temperatures and was not corrected for high viscosity.

As described in Materials in Methods, absolute rates of enzymatic catalysis usually depend on the solvent viscosity. The first attempts to include viscosity into the Kramers' approach to describe biochemical reactions yielded an inverse dependence of the rate of a reaction on viscosity (i.e. $p = 1$)²⁸. This approach was tested for hydrolysis catalyzed by carboxypeptidase²⁹, and later for the variety of processes such as oxygen escape from myoglobin³⁰ and hemerythrin³¹, ester hydrolysis by subtilisin³², photocycle of bacteriorhodopsin³³ and hydrogen isotope exchange in lysozyme and in other proteins^{34,35}. In all these experiments it was observed that biochemical reactions that take place within a protein matrix are sensitive to the viscosity of the solvent, especially at high viscosities, and that in most cases a fractional power ($p < 1$) describes the process. In a systematic study of many solvents, Yedgar et al.²⁵ have shown that the effect of solvent viscosity on the escape of oxygen from hemerythrin follows the power-law with p dependent on the molecular weight of the cosolvent. This relation can be expressed as:

$$p(M)=1.52M^{-0.23} \quad (3)$$

where, M is the molecular weight (in Daltons) of the cosolvent. For glycerol, $M = 92$ and therefore, $p = 0.54$. This empirical value is also the value that the authors determined experimentally.

Temperature dependence of viscosity

Our TDS experiment samples temperatures from 230K to 290 K. In this range, the viscosity of water-glycerol mixtures strongly depends on temperature²⁶. There are various models that attempt to correlate viscosity-temperature data of pure liquids and liquid-solutions starting from the simple Arrhenius type models³⁶ to more elaborate and complex empirical fits^{37,38}. Most of these models have been derived from fitting experimental data for the viscosity of aqueous glycerol solutions at temperatures between 253 K and above. These models often fail when used to make predictions outside their validity limit. We used the equation derived by Trejo González *et al.*²⁶, which uses the general Aramov-Michev equation³⁹ to model viscosity data of glycerol aqueous solutions in the entire range of compositions, $0 \leq w_2 \leq 1$ and which also provides equations for the composition dependence of the parameters in this equation. This approach was also shown to be applicable over a wide range of temperatures including the supercooled region of water and glycerol where other methods of predicting the viscosity-temperature relationship fail. The equations used for calculating viscosity of the glycerol-water mixture used are:

$$\eta=\eta^0\exp\left[(28.75-\ln\eta^0)\left(\frac{T_g}{T}\right)^\alpha\right] \quad (4)$$

Where, T_g is the glass transition temperature of the liquid solution. η_0 , T_g and α were expressed as a functions of the weight fraction of glycerol (w_2) in the solution as:

$$T_g(K)=140.3+35.272w_2 - 3.879w_2^2+23.467w_2^3 \quad (5)$$

$$-\ln(\eta_0/mPa \cdot s)=2.1686 - 2.7096w_2+1.4449w_2^2 \quad (6)$$

$$\alpha=3.6344+1.2198w_2 - 1.6142w_2^2 \quad (7)$$

If not corrected for, temperature dependence of η contributes ~ 5 kcal/mol to the observed energy barrier as seen by Gavish and Werber while studying the rate of catalysis of carboxypeptidase in different cosolvent mixture²⁹. Fitting the TDS data reported in this paper to the simple Arrhenius equation, not accounting for viscosity of solution or its temperature dependence, yields the same result.

A value of 0.65 was used as the weight fraction of glycerol instead of 0.75 that would result if one calculated the weight fraction of glycerol in a 70% (v/v) glycerol-water mixture. The presence of glycerol preferentially hydrates protein molecules and results in a higher than average content of water in the hydration shell of the protein⁴⁰. Preferential hydration in the presence of glycerol and ethylene glycol is known to stabilize the activity of enzymes and retain the native structure of proteins⁴¹. For a 75% w/w glycerol-water mixture the amount of glycerol present in the local domain of the protein molecule is approximately 65%⁴⁰.

Intracellular microviscosity is an important regulator of cellular functions and membrane enzyme activity⁴². The analysis developed in this work, integrating the two important parameters of temperature and viscosity, not only gives a full Arrhenius dependence of the also provides a way to calculate rates of autoxidation of oxy-P450s in different cellular milieu. Importantly, high viscosity has a profound effect on the rates of autoxidation, and in some cases this can affect the stability of oxy-ferrous intermediate in membrane bound cytochromes P450 and overall uncoupling of P450 catalysis.

Apart from being quick, this technique offers high precision, in terms of agreement between experimental data and the theoretical model of autoxidation decay, as well as high accuracy, as evident from the comparison of E_a values obtained with this methodology and that obtained by other approaches. TDS gives spectral data corresponding to a finely spaced, wide range of temperatures, the eigenvalues and eigenvectors obtained from SVD analysis are more robust than that obtained from the SVD analysis of spectral data sets at five to six temperature points in a typical stopped-flow experiment. TDS also gives the complete Arrhenius dependence of the autoxidation process in a single experiment, using very little sample.

CONCLUSION

TDS provides a rapid, robust and a convenient method to measure decay kinetics of oxy-ferrous complexes in cytochromes P450. Using this method, the full Arrhenius dependence of the autoxidation process can be measured in a single experiment, with relatively little sample. Furthermore, the sample is completely recoverable unlike in stopped-flow. By integrating data analysis with data acquisition the method described has the potential to be developed into a semi-automatic way to characterize the oxy-ferrous complex in heme proteins. The concept, in general, can be applied to any temperature-dependent process, the

‘observable’ varying from absorption spectra, as illustrated here, to other parameters such as mid-point reduction potential and pH.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

The catalytic cycle of the cytochromes P450 showing the various iron oxygen intermediates and the three unproductive shunt pathways.

Figure 2.

Theoretical curves depicting the effect of temperature ramp rate on the shape of the concentration profile. All curves were generated assuming an 18 kcal/mol Arrhenius activation energy.

Figure 3a

Figure 3b

Figure 3c

Figure 3.

Figure 3a. SVD reconstructed TDS data for AD-bound-CYP19. *Inset:* Difference spectra corresponding to the same data set. Both set of spectra show clear transition from the oxy-ferrous complex to the ferric resting state.

Figure 3b. Eigenvectors from singular value decomposition (SVD) of TDS data for AD-bound-CYP19, normalized with respect to the corresponding eigenvalues.

Figure 3c. Decomposition of the oxyferrous complex of AD-bound-CYP19 via autoxidation fitted to a rate equation based on Kramers' theory with viscosity corrections. The up-tick towards the end of the experiment, as seen in the figure, could be due to light scattering. Condensation of water molecules on the walls of the cuvette, changes in turbidity of the sample solution with rise in temperature contribute to this effect.