

The Effect of Solution Viscosity on Intramolecular Electron Transfer in Sulfite Oxidase[†]

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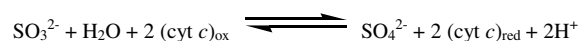
¹Abbreviations: SO, sulfite oxidase; (cyt *c*)_{ox} and (cyt *c*)_{red}: ferricytochrome *c* and ferrocycytochrome *c*, respectively; dRF and dRFH•: 5-deazariboflavin and 5-deazariboflavin semiquinone, respectively; EDTA, ethylenediaminetetraacetic acid; PEG-400, polyethylene glycol-400; IET, intramolecular electron transfer; k_{et} , rate constant for intramolecular electron transfer; η , absolute viscosity.

ABSTRACT

Our previous studies have shown that the rate constant for intramolecular electron transfer (IET) between the heme and molybdenum centers of chicken liver sulfite oxidase varies from about 20 – 1500 s⁻¹ depending upon reaction conditions [Pacheco, A., Hazzard, J. T., Tollin, G., Enemark, J. H. (1999) *JBIC* 4, 390-401]. These two centers are linked by a flexible polypeptide loop, suggesting that conformational changes that alter the Mo ... Fe distance may play an important role in the observed IET rates. In this study we have investigated IET in sulfite oxidase using laser flash photolysis as a function of solution viscosity. The solution viscosity was varied over the range 1.0 – 2.0 cP by addition of either polyethylene glycol-400 or sucrose. In the presence of either viscogen, an appreciable decrease in the IET rate constant value is observed upon an increase in the solvent viscosity. The IET rate constant exhibits a linear dependence on the negative 0.7th power of the viscosity. Steady-state kinetics and EPR experiments are consistent with the interpretation that viscosity, and not other properties of the added viscogens, is responsible for the dependence of IET rates on the solvent composition. The results are consistent with the role of conformational changes on IET in sulfite oxidase, which helps to clarify the inconsistency between the large rate constant for IET between the Mo and Fe centers and the long distance (32 Å) between these two metal centers observed in the crystal structure [Kisker, C., Schindelin, H., Pacheco, A., Wehbi, W., Garnett, R. M., Rajagopalan, K. V., Enemark, J. H., Rees, D. C. (1997) *Cell* 91, 973-983].

Introduction

Sulfite oxidase (SO) catalyzes the oxidation of sulfite to sulfate, coupled with the subsequent reduction of two equivalents of ferricytochrome *c* (cyt *c*)_{ox} to ferrocycytochrome *c* (cyt *c*)_{red} (1):



This is the terminal reaction in the oxidative degradation of sulfur-containing compounds, and is physiologically essential. In animals the enzyme is located in the mitochondrial intermembrane space (2, 3); the most extensively studied examples of SO are from rat, human, and chicken livers, and all of these show a very high degree of amino acid sequence homology (4-6). Chicken liver SO is typical of the enzyme in higher vertebrates, and consists of two identical subunits, each with a molecular weight of ~51.5 kDa (7). Each subunit has two functionally distinct domains. The smaller N-terminal domain (~10 kDa) is typical of a small *b*₅-type cytochrome, containing a non-covalently bound heme cofactor; in the resting enzyme, the heme iron is in the oxidation state of Fe^{III}. The larger C-terminal domain (~42 kDa) contains the Mo-pterin cofactor and the Mo is in the resting oxidation state of Mo^{VI}.

FIGURE 1 summarizes the oxidation state changes that are thought to occur at the Mo and Fe centers during the catalytic cycle (8). The cycle begins with the 2-electron oxidation of SO₃²⁻ by the Mo^{VI}, which is not detectable by spectroscopy. The first species that is spectroscopically detected is Fe^{II}Mo^V, probably generated by direct intramolecular electron transfer (IET) from the molybdenum to the iron center. Reoxidation of the Fe^{II} center occurs by one-electron transfer to cytochrome *c*, leaving the enzyme as Fe^{III}Mo^V. A second Mo to Fe IET step (giving Fe^{II}Mo^{VI}), followed by reduction of a second equivalent of cytochrome *c*, returns the enzyme to the resting state (Fe^{III}Mo^{VI}). Note that equilibrium between Fe^{II}Mo^{VI} and Fe^{III}Mo^V is established by the second IET step, with rate constants *k*_f and *k*_r for the forward and reverse reactions, respectively. These rate constants have been found to be affected

by various parameters such as pH, anion concentration, and even the nature of the anion; thus, Cl⁻, SO₄²⁻, PO₄³⁻ or EDTA all have different inhibitory effects (7-9).

In this study, as well as the previous reports, we have used the flash photolysis technique to generate the Fe^{II}Mo^{VI} form of SO and to measure k_f and k_r under various conditions, as shown in FIGURE 1 (7, 9). Using this technique, one directly obtains values for the intramolecular electron transfer rate k_{et} ($= k_f + k_r$) and the equilibrium constant K_{eq} ($=k_f/ k_r$) as described previously (9).

Under optimal conditions, the value of k_{et} has been measured to be 1400 s⁻¹ (7). In the X-ray crystal structure of SO, the Mo and Fe centers are 32 Å apart (10). Based on current models for IET over such a large distance, the rate constant should be much less than this (#100 s⁻¹) (11). This indicates that the position and orientation of the redox partners with respect to each other observed in the crystal structure is less than optimal for electron transfer (7). One possible explanation for this discrepancy is that the protein conformation during electron transfer in solution is different from that seen in the crystal structure. Rearrangement to a more “productive” orientation may occur before electron transfer, which suggests that fast electron transfer between Mo (VI) and Fe (II) centers requires subtle and precise positioning and orientation of the two redox partners with respect to each other. FIGURE 2 shows a schematic drawing of the relative positions of the Mo and Fe domains in the crystal structure, and depicts how the backbone rearrangement of the protein might allow closer contact between the two metal centers before the electron transfer takes place. The Mo-pterin and heme domains of SO are linked by a very flexible loop consisting of 10 amino acid residues (10), which could provide the heme domain with the necessary mobility to allow its negatively charged exposed edge to interact electrostatically with the positively charged Mo site. The existence of such an inter-domain flexible loop makes SO a unique candidate for studying role of protein re-arrangement in electron transfer in biological macromolecules.

It is generally accepted that the structural dynamics of proteins in solution plays an important role in regulating their biochemical function. The interaction of the protein structure with the surrounding water is well known to be intimately linked with this type of structural dynamics, and Brownian theory indicates that the internal friction associated with such molecular motion, which is linked to the solvent viscosity, should affect protein dynamics. The role of solvent viscosity in controlling chemical kinetics was proposed by Kramers in 1940 (12), and the applicability of Kramers’ theory to protein reactions was demonstrated by Gavish in 1978 and 1979 (13, 14). Since then, a large number of studies of solvent viscosity effects on biochemical kinetic processes have been carried out, and in most cases the observed rate constant k_{obs} has been shown to be inversely proportional to the fractional power (α) of the viscosity η (Eq. 1) (15).

$$k_{obs} = B\eta^{-\alpha} \quad (0 < \alpha \leq 1) \quad (1)$$

Examples include peptide hydrolysis by carboxypeptidase A (16), binding of O₂ or CO to respiratory proteins (17), electron transfer in inter-protein complexes (18), as well as protein conformational changes (19), protein dynamics (20) and protein folding (21). To the best of our knowledge, very few studies of the effect of viscosity on intramolecular electron transfer in proteins have been reported. In order to determine if conformational changes in SO have an effect on IET, herein we have measured the effect of solution viscosity on the IET rate constant (k_{et}) using the laser flash photolysis technique.

EXPERIMENTAL PROCEDURES

Materials. PEG 400 and sucrose were obtained from Sigma Chemical Co. Distilled water was demineralized to a resistance greater than 18 M Ω cm. Horse-heart cytochrome *c* (Type VI) was purchased from Sigma Chemical Co. The method used to purify chicken SO has recently been described in detail (10). Fractions with $A_{414} / A_{280} > 0.93$ were pooled together and used for flash photolysis.

Viscosity. The absolute viscosity (η) of water and of aqueous solutions of sucrose were taken from tables (22). The relative viscosity (η/η_0) of buffers with and without PEG 400 was measured with a glass viscometer at 293 K; the absolute error was ± 0.05 cP. Given $\eta_0 = 1.002$ cP, the absolute viscosity (η) was calculated. The contribution of salts to viscosity was neglected. The contribution of proteins to viscosity, since they were present only at micromolar concentrations, was also neglected.

Laser Flash Photolysis Studies. 5-Deazariboflavin (dRF), a photoactive species, is excited with a laser pulse, which results in the formation of a semiquinone (dRFH \cdot) in the presence of a sacrificial donor such as EDTA or semicarbazide. The semiquinone radical is a strong reducing agent, and rapidly reduces SO to Fe^{II}Mo^{VI}. The ensuing slower equilibrium between Fe^{II}Mo^{VI} and Fe^{III}Mo^V can subsequently be monitored, by measuring the absorbance change at 555 nm, as shown in FIGURE 3 (see below). Laser flash photolysis experiments were performed anaerobically on 0.50 ml solutions containing approximately 90 μ M dRF and 0.5 mM semicarbazide as a sacrificial reductant in 10 mM Tris buffer solution (pH 7.4). The concentration of the inhibitory chloride anion was 6.5 mM. The viscosity of the solutions was adjusted by adding an appropriate amount of buffered solutions, at the same pH and ionic strength, of sucrose or PEG 400. Final concentrations of PEG 400 in volume percentage and sucrose in mass percentage are 0%–10% and 0%–20%, respectively. Buffers with PEG 400 or sucrose were deaerated by vigorous bubbling with argon overnight. Enzyme concentrations were determined by using molar extinction coefficients of 99,900 M⁻¹ cm⁻¹ at 413 nm for the oxidized chicken SO. The methodology used for flash photolysis has been described previously (9). The laser apparatus and associated visible absorbance detection system have been extensively described (23), as has the basic photochemical process by which 5-deazariboflavin semiquinone is generated by reaction between triplet state dRF and the sacrificial reductant semicarbazide and used to reduce redox-active proteins (24, 25, 26). Further details concerning the photochemical process, which are of particular relevance to the SO system, are presented below.

Non-linear least squares fitting of transient experimental data obtained at 513 nm was generally performed using an implementation of the Levenberg-Marquart algorithm, provided as part of the Microcal Origin (version 6.1; Northampton, MA) software package for data processing and display. Transient absorbance changes obtained at 555 nm were analyzed using the computer fitting procedure SIFIT, obtained from OLIS Inc. (Jefferson, GA).

Steady-state Kinetics Studies. Steady-state enzyme kinetics were performed aerobically in a Varian Cary-300 spectrophotometer, using saturating (10-fold greater than the corresponding K_m) concentrations of sulfite (220 μ M), and varying the concentration of cytochrome *c*. Initial velocities were determined by following the reduction of a freshly prepared oxidized cytochrome *c* solution at 550 nm, using an extinction coefficient change of 19630 M⁻¹ cm⁻¹ (27). Experiments were carried out in 100 mM Tris buffer, adjusted to pH 8.0 with acetic acid in order to minimize the possibility of anion inhibition.

RESULTS AND DISCUSSION

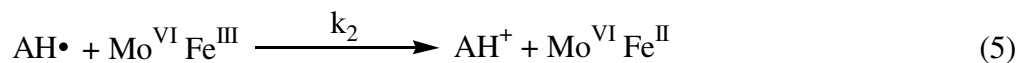
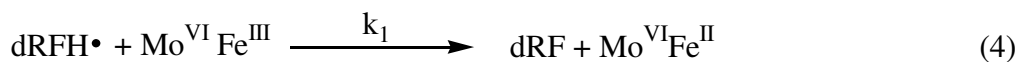
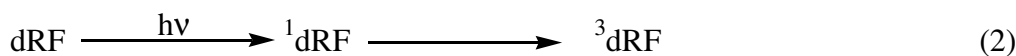
Steady-state Kinetics of Sulfite Oxidase in the presence of PEG 400

The steady-state oxidation of sulfite to sulfate as catalyzed by SO using cytochrome c as the electron acceptor yields plots of initial velocity versus substrate concentration that display typical saturation kinetics (FIGURE 4A). The k_{cat} and $K_m^{(cyt\ c)}$ values obtained from these experiments for SO with 0 %, 10 %, 20 % (v/v) PEG 400 are listed in Table 1. Within experimental error, these data indicate that PEG-400 has at best a minor effect on the activity (i.e., k_{cat}) of SO in the range of viscosity studied (FIGURE 4B).

Effects of Viscosity on Electron Transfer rates in SO

FIGURE 3 shows a typical transient kinetic trace obtained at 555 nm upon laser flash photoexcitation of a solution containing oxidized chicken SO, 5-deazariboflavin, semicarbazide and 5 % (v/v) PEG 400. The transient absorbance changes observed at this wavelength are directly related to reduction and re-oxidation of the *b*-type heme prosthetic group (7). As expected, no detectable spectral contribution from the Mo cofactor was observed. It is important to note that SO in the presence of PEG 400 or sucrose has similar photochemical reduction properties as SO in the absence of these viscogens.

The kinetic behavior can be fully described in terms of the minimal set of reactions shown in equations 2 – 6 below. Deazariboflavin semiquinone (dRFH•) is generated by the laser pulse in the presence of the sacrificial electron donor semicarbazide (AH₂) (Eq. 3). The initial positive deflection of absorbance from zero in FIGURE 3 is due to net reduction of the SO heme center to the Fe^{II} form (Eq. 4), which has an absorbance maximum at 555 nm. The slow decrease in absorbance that follows the initial rapid increase is due to the net IET from Fe^{II} to Mo^{VI}, which establishes equilibrium between the Mo^{VI}Fe^{II} and Mo^VFe^{III} forms of the enzyme (Eq. 6). The kinetics of this latter process is independent of the concentration of the enzyme within the range of viscogen concentrations used, indicating that it is still due to a first-order intramolecular electron transfer process in the presence of both PEG 400 and sucrose, which is the reaction that is of particular interest to us in this study. The IET rate constant is designated k_{et} , and is the sum of the forward (k_f) and reverse (k_r) electron transfer rate constants (see FIGURE 1 and Eq. 6). This decay curve also provides a means of determining the equilibrium constant K_{eq} ($= k_f / k_r$) for this IET process from the extent of the absorbance decay. To minimize the contribution of AH• reduction of Fe^{III} (Eq. 5) and its corresponding spectral overlap with the IET process, semicarbazide was used as a donor in the present work instead of EDTA (9).



The observed rate constant for the initial heme reduction is protein concentration dependent, as expected for a bimolecular process (Eq. 4). Electron transfer from dRFH• to the heme of sulfite oxidase can best be observed spectrophotometrically as a decrease in absorbance at 513 nm, which is close to the absorbance maximum for dRFH• and an isosbestic point for oxidized and reduced heme

cofactor. Increasing the viscosity by addition of sucrose decreased the second-order rate constant (k_1) for electron transfer between heme b_5 Fe (III) and the radical dRFH•, from $1.57 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$ at 1.0 cP to $1.20 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$ at 1.9 cP (data not shown). Increasing the viscosity with PEG 400 had a similar effect. These results are as expected since this heme reduction reaction has a rate constant close to the diffusion-controlled value.

FIGURE 5A shows that the values of k_{et} decrease progressively with increase of viscosity. In FIGURE 5B, normalized values of the IET rate constants are plotted, where $k_{\text{et } 0}$ denotes the IET rate constant in the absence of viscogen. All normalized values, determined in buffered mixtures of water with the two different solutes, PEG 400 and sucrose, fall on the same line when plotted versus the viscosity. It is important to note that recent studies show that the magnetic circular dichroism heme spectra do not change in the presence of PEG-400, suggesting that the heme environment is not affected by this polymer (28). Furthermore, the CW-EPR spectrum of sulfite-reduced SO in 20 % sucrose is the same as that without sucrose (data not shown), which indicates that the active site structure of the molybdenum domain remains unchanged upon addition of sucrose. In combination with the steady-state kinetics studies described above, we conclude that it is solution viscosity, and not other properties of the added viscogens and/or their effect on SO structure, that is responsible for the dependence of IET rates on the solvent composition.

The viscosity dependence of k_{et} was fitted to the modified Kramers equation (Eq. 1) (see FIGURE 6), which shows that that the IET rate constant in SO has a linear dependence on the negative 0.7th power of the viscosity in the presence of either viscogen. Because this electron transfer rate constant is independent of protein concentration and is thus due to an intramolecular process, the most plausible explanation for the significant viscosity dependence behavior is that a large conformational change precedes the intramolecular electron transfer in SO. Furthermore, the conformational change must be large enough so that the intramolecular movement of the protein is impeded by the solvent. These results indicate that the observed IET rate is not due to the intrinsic electron transfer process, but rather is gated by a protein conformational change, presumably mediated by the domain-connecting loop in SO. Further experiments in which this loop is shortened by site-directed mutagenesis are underway and will provide more direct evidence for the role of the flexibility of this portion of the molecule in electron transfer in SO.

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FIGURE CAPTIONS

FIGURE 1. Oxidation state changes at the Mo and Fe centers of SO during the catalytic oxidation of sulfite, and concomitant reduction of cyt *c*. Flash photolysis provides a way to reduce SO by one electron (shown by a *dashed arrow* connecting $\text{Mo}^{\text{VI}}\text{Fe}^{\text{III}}$ to $\text{Mo}^{\text{VI}}\text{Fe}^{\text{II}}$) in a solution containing 5-deazariboflavin (dRF) and the sacrificial electron donor semicarbazide. The ensuing equilibrium can thus be observed without creating the intermediate species in the cycle. The rate constants k_f and k_r are defined in the text.

FIGURE 2. Proposed conformational change in sulfite oxidase that could move the Mo and Fe centers into closer proximity than that observed in the crystal structure. Only one subunit of SO is portrayed for clarity.

FIGURE 3. Transient kinetic trace obtained at 555 nm upon photoexcitation of a solution containing 24.0 μM chicken SO, $\sim 90 \mu\text{M}$ dRF, 5 % (v/v) PEG 400 and 0.5 mM semicarbazide in 10 mM Tris buffer (pH 7.4). The solid line indicates a single-exponential fit to the IET phase.

FIGURE 4. (Panel A), Hyperbolic plots of values of initial velocities divided by total concentration of SO (1.1×10^{-10} M) versus varying concentrations of cyt *c*. Reaction conditions were 0.1 M Tris-acetate buffer, pH 8.0, 25 °C; (Panel B), Plot of k_{cat} values versus concentration of PEG 400.

FIGURE 5. (Panel A), Viscosity dependence of the rate constant k_{et} for intramolecular electron transfer in sulfite oxidase, shown in Eq. 6, in Tris buffer at pH 7.4 using sucrose as viscogen; (Panel B), Plot of normalized values ($k_{\text{et}0} / k_{\text{et}}$), in which $k_{\text{et}0}$ denotes the rate constant in the absence of viscogens, versus viscosity, in PEG 400 (closed symbols) and sucrose (open symbols). As is evident, the two data sets can be fit by the same line.

FIGURE 6. Fit of viscosity dependence of the rate constant k_{et} in PEG 400 (closed symbols) and sucrose (open symbols) using Eq. 1.

Table 1. k_{cat} and $K_m^{(\text{cyt } c)}$ values for sulfite oxidase versus volume percentage of PEG-400

PEG % (v/v)	Viscosity (cP)	k_{cat} (s^{-1})	$K_m^{(\text{cyt } c)}$ (μM)
0	1.002	73.2 ± 3.0	4.3 ± 0.6
10	1.653	79.6 ± 5.7	5.4 ± 0.8
20	2.735	76.0 ± 2.6	3.3 ± 0.3

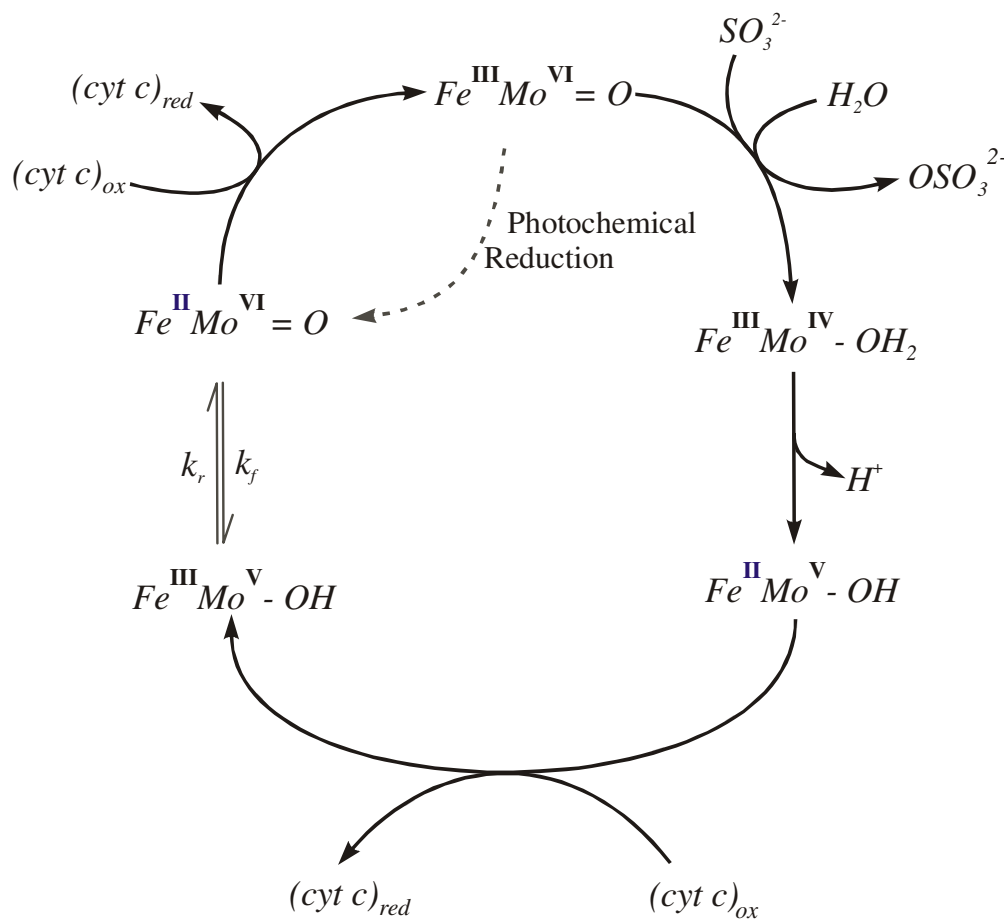


FIGURE 1

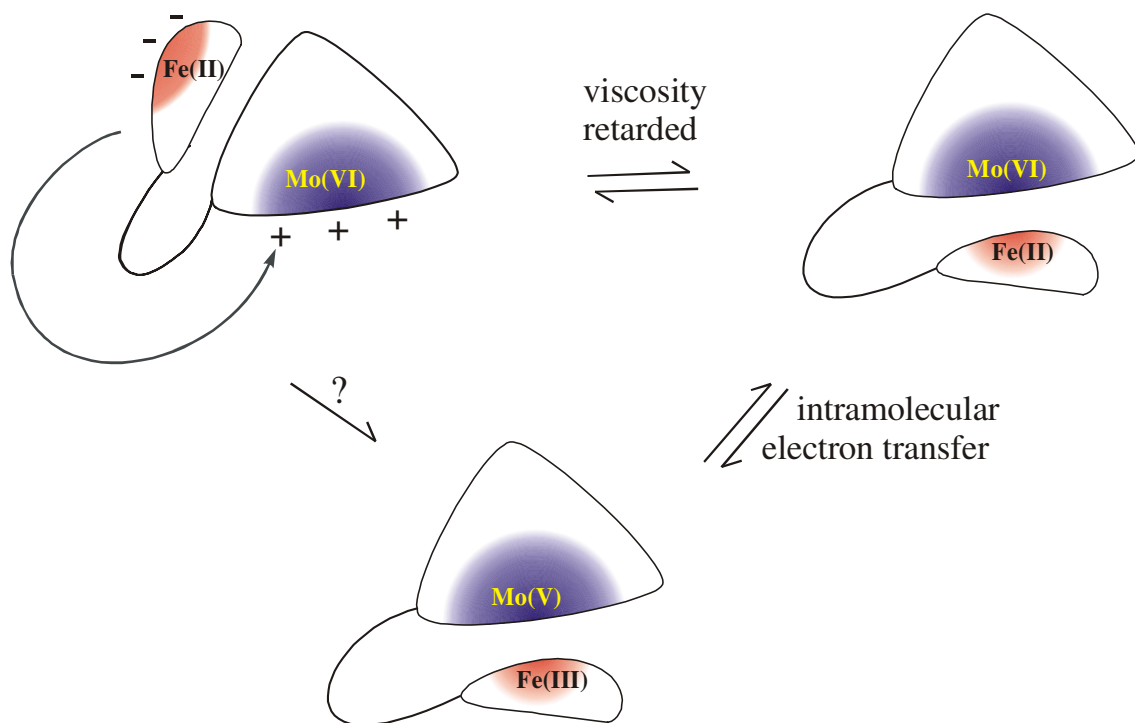


FIGURE 2

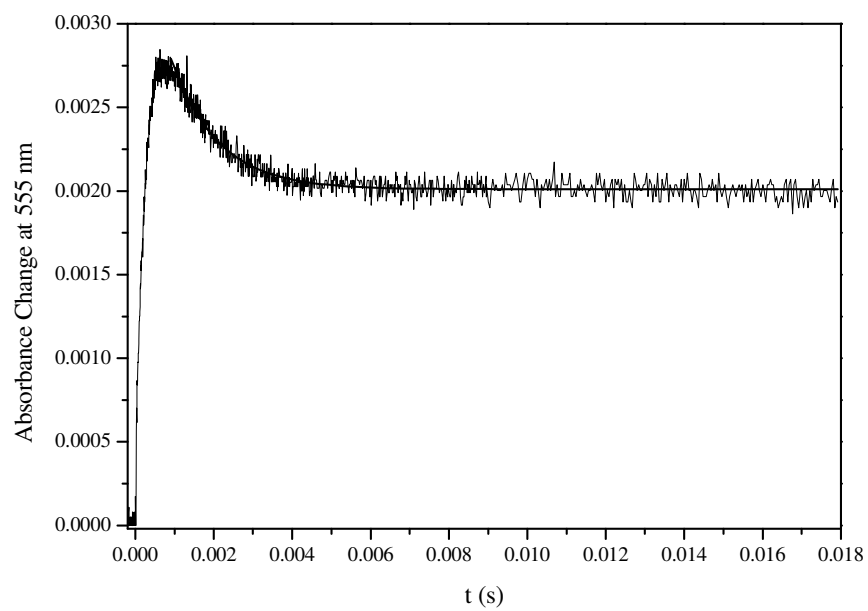


FIGURE 3

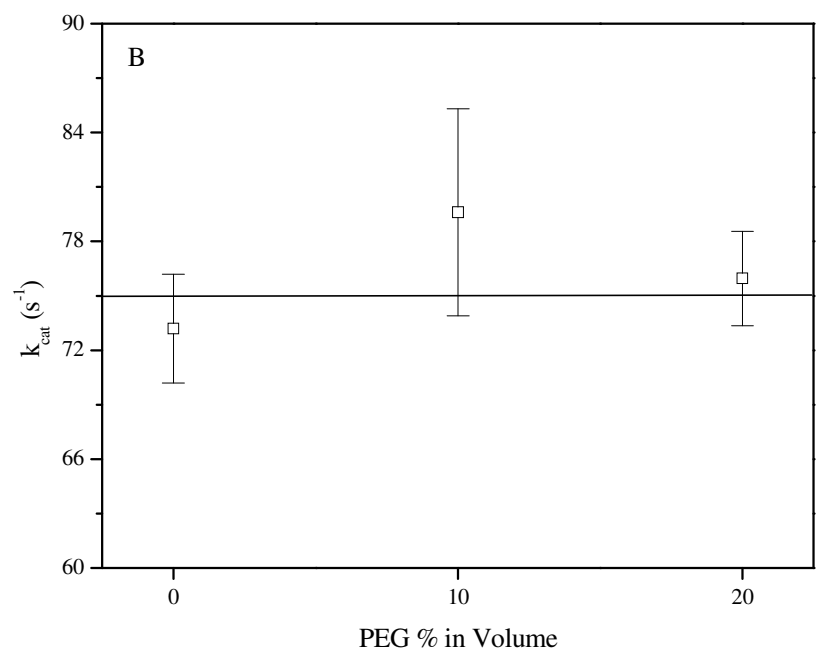
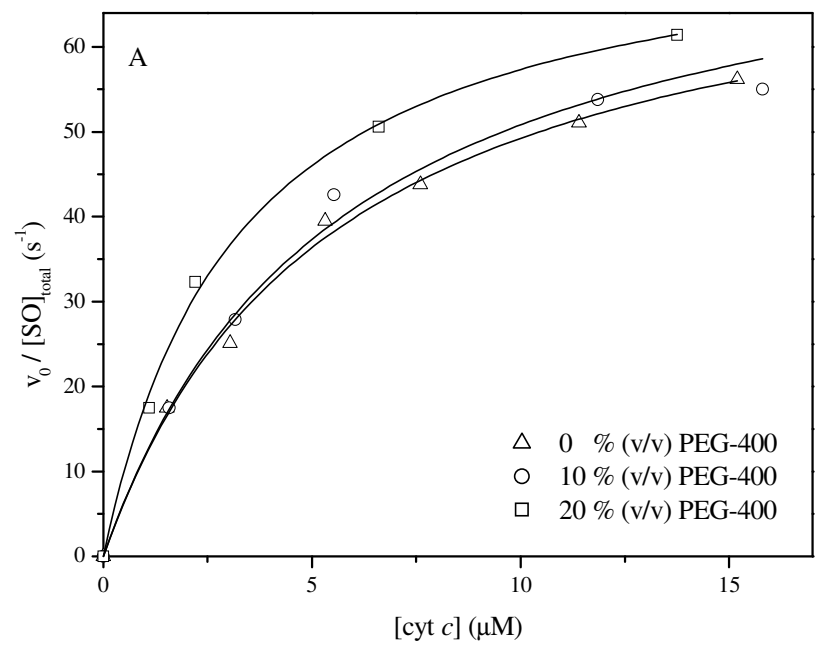


FIGURE 4

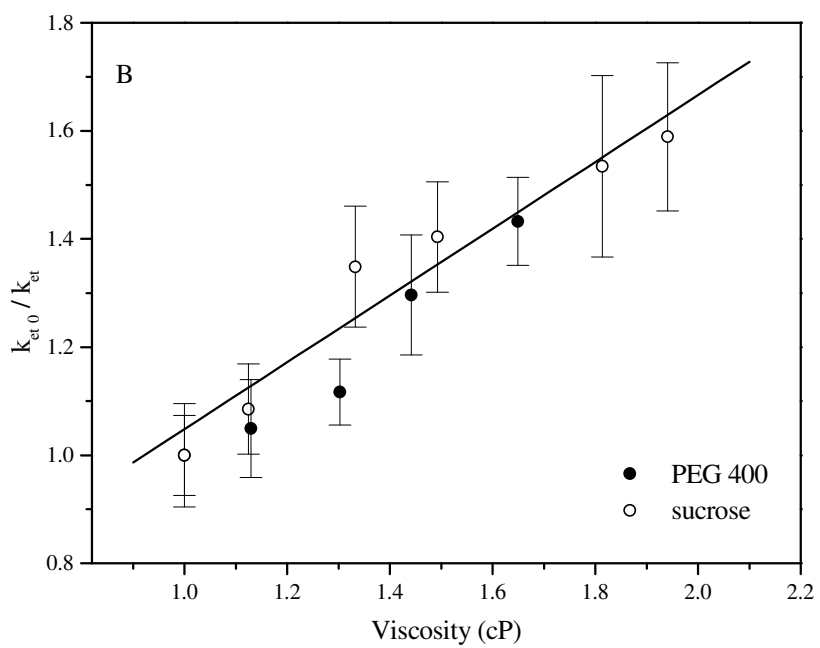
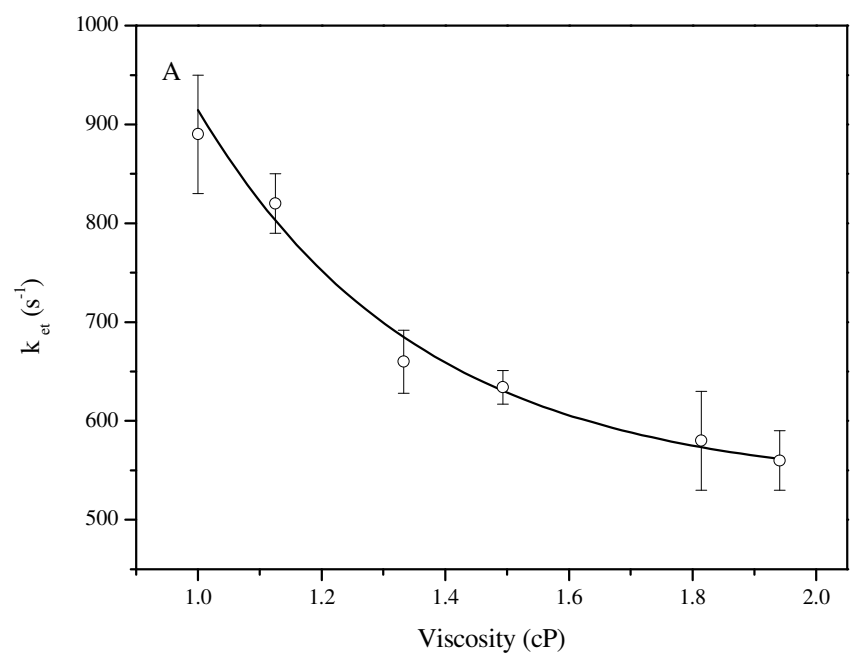


FIGURE 5

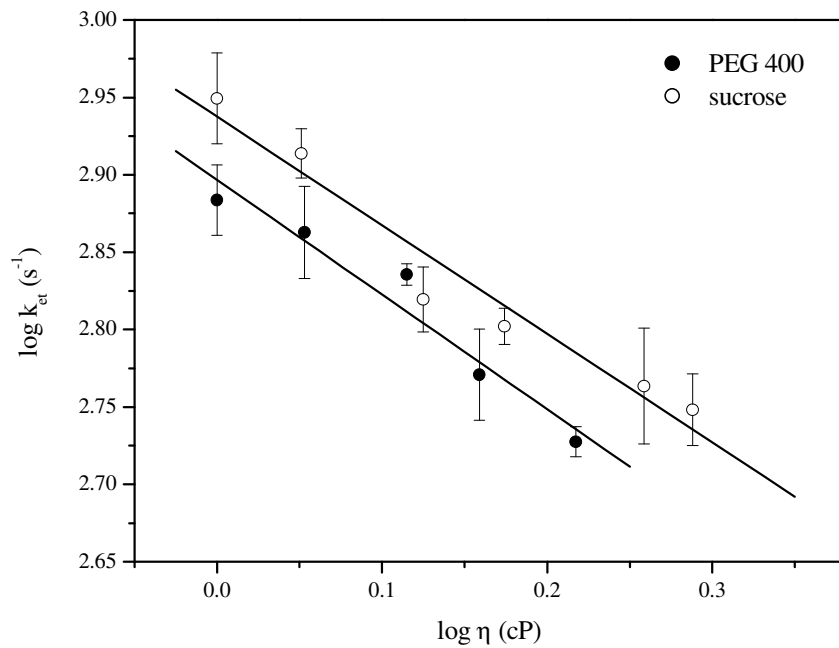


FIGURE 6