Monooxygenase-like Activity of Methemoglobin with Sodium Sulfite as an Efficient Reductant

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An important goal of protein engineering is the introduction of useful activities into readily available proteins. Cytochrome P-450 systems catalyze the insertion of an oxygen atom into C–H bonds of organic compounds using dissolved dioxygen which is cleaved and reduced (Scheme I).[1-3]

The general utility of such systems is limited by the availability of P-450s, the need for a reductase, and the stoichiometric involvement of NADPH, which serves as a reducing agent. Limitations due to protein availability can be circumvented by introducing the desired activity into a readily available protein. Mieyal found that an abundant protein, methemoglobin, can substitute for cytochrome P-450 in a system which utilizes NADPH as an electron source and cytochrome P-450 reductase to mediate electron transfer.[4-6] In an effort to simplify the system, Kaiser coupled a flavin to hemoglobin.[7] This functioned efficiently without a reductase, but its utility was limited by the need for NADPH and the complex preparation of flavohemoglobin. A more practical solution requires replacement of the reductase and NADPH in a system utilizing hemoglobin and oxygen. We now report that sodium sulfite efficiently replaces both NADPH and reductase in promoting the monooxygenase activity of methemoglobin. The reaction patterns are consistent with sequential one-electron transfers with intermediate formation of the sulfite monoanion radical bound to Fe(II) hemoglobin.

Catalysis of the conversion of aniline to *p*-aminophenol by methemoglobin (prepared by ferricyanide oxidation of hemoglobin) tests monooxygenase activity.[5] We conducted the reaction in 20 mM potassium phosphate buffer (pH 7.0) with varying concentrations of sodium sulfite (under atmospheric O_2 at 37 °C). The assay is linear over the 15-min period of observation and is proportional to methemoglobin concentrations to 1.0 μM. During the assay, hemes within the protein tetramer show an unchanged Soret band, characteristic of the native protein. This contrasts with reactions which produce a destructive oxidant.[8] The cleavage of anisole is a more demanding test of monooxygenase activity.[9,10] GC–MS analysis of the reaction of anisole showed efficient production of phenol and formaldehyde (140 pmol/min phenol and formaldehyde; 1 mL of pH 7.0 KP_i, 2.0 mM anisole, 1 μM methemglobin, 0.6 mM sodium sulfite).

The catalytic process is inhibited by CO, implicating hemoglobin-Fe(II) as an intermediate, to which CO binds strongly, blocking the normal dioxygen-activation processes. The reaction with oxygen is not subject to inhibition by hydroxyl radical scavengers: ethanol (1 M), mannitol (0.1 M), or DMSO (0.1 M). Superoxide and peroxide are not involved in the hydroxylation process since superoxide dismutase (up to 40 μg/mL) and catalase (up to 20 μg/mL) have no effect. An Fe^{V}=O intermediate (Scheme I) reacting directly with bound aniline would account for these observations. Addition of peroxysulfate in place of sulfite allows the reaction to function anaerobically (full activity under nitrogen and continued oxidation in the presence of CO), consistent with the implied peroxy shunt in a cyclic mechanism.[11] In the absence of methemoglobin, peroxymonosulfate reacts slowly with aniline and gives different products.

The hydroxylation follows Michaelis–Menten kinetics with respect to aniline at constant sodium sulfite concentration.

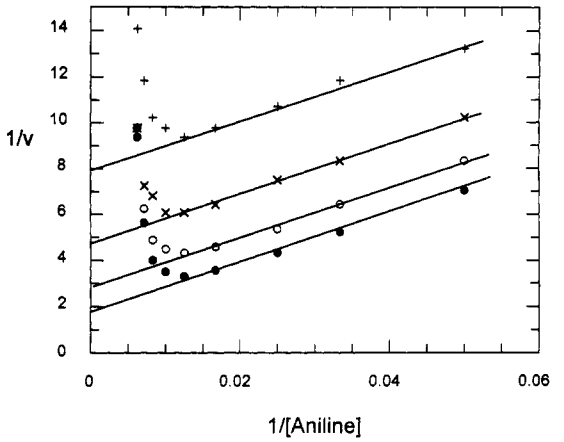
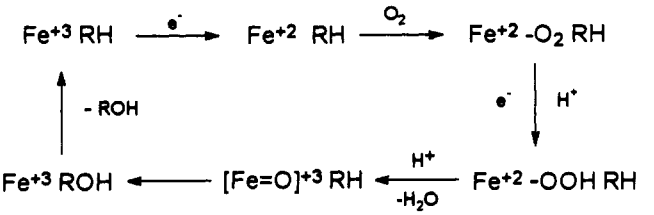
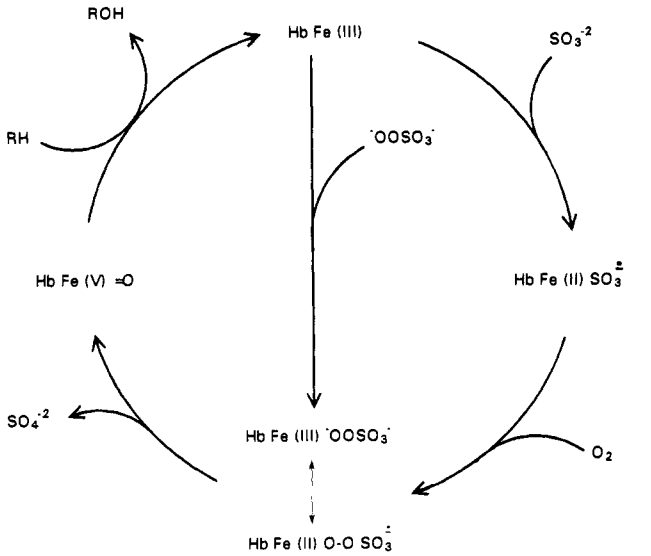


Figure 1. Combined Lineweaver–Burk plots of rates of *p*-hydroxylation of aniline (mM) as a function of sodium sulfite concentration: 0.05 (+), 0.10 (×), 0.20 (○), and 0.40 mM (●). Reactions were initiated by addition of sodium sulfite, allowed to proceed at 37 °C for 15 min, and terminated by addition of 20% trichloroacetic acid.

Scheme I



Scheme II



(1) Abeles, R. H.; Frey, P. A.; Jencks, W. P. *Biochemistry*; Jones and Bartlett: Boston, 1992.
(2) Ortiz de Montellano, P. R., Ed. *Cytochrome P-450: Structure, Mechanism, and Biochemistry*; Plenum: New York, 1986.
(3) Fox, B. G.; Borneman, J. G.; Wackett, L. P.; Lipscomb, J. D. *Biochemistry* **1990**, *29*, 6419.
(4) Mieyal, J. J.; Ackerman, R. S.; Blumer, J. L.; Freeman, L. S. *J. Biol. Chem.* **1976**, *251*, 3436.
(5) Ferraiolo, B. L.; Onady, G. M.; Mieyal, J. J. *Biochemistry* **1984**, *23*, 5528.
(6) Starke, D. W.; Mieyal, J. J. *Biochem. Pharmacol.* **1989**, *38*, 201.
(7) Kokubo, T.; Sassa, S.; Kaiser, E. T. *J. Am. Chem. Soc.* **1987**, *109*, 606.
(8) Galaris, D.; Sevanian A.; Cadenas, E.; Hochstein *Arch. Biochem. Biophys.* **1990**, *281*, 163.
(9) Lindsay Smith, J. R.; Piggott, R. E.; Sleath, P. R. *J. Chem. Soc., Chem. Commun.* **1982**, 55.
(10) Starke, D. W.; Blisard, K. S.; Mieyal, J. J. *Mol. Pharmacol.* **1984**, *25*, 467.
(11) Porter, T. D.; Coon, M. J. *J. Biol. Chem.* **1991**, *266*, 13469.

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Variation of sodium sulfite concentration produces kinetic plots indicating a ping-pong mechanism (Figure 1). High concentrations of aniline (>80 mM) give substrate inhibition.[12]

Scheme II is based on analogy to the mechanisms of dioxygen activation by P-450,[2,11] the mechanism of reduction of cytochrome *c* by sulfite,[13] and the kinetic and inhibition patterns above.[12] Methemoglobin binds sulfite to form a complex in which electron transfer gives the sulfite anion radical bound to ferrous hemoglobin, to which oxygen then binds. This can be considered to be a resonance structure of a methemoglobin complex of peroxysulfate. Cleavage of sulfate leaves $Hb^{V}{=}O$, the proposed active species in oxygen insertion reactions. Oxygen is inserted into bound substrate, regenerating methemoglobin.

The kinetic data for the aniline reaction were fit to equations for ping-pong kinetics:[12] $k_{cat} = 0.015\ s^{-1}$, K_m(aniline) = 91 mM, and K_m(sodium sulfite) = 0.35 mM. The K_m for aniline is similar to that with the α subunits of Hb in the reductase-promoted hydroxylation (102 mM) and higher than that reported with the β subunits.[5] Unlike the association of P-450 reductase with hemoglobin, sulfite should be attracted to cationic sites such as the ferric ions in methemoglobin. In addition, k_{cat} ($0.015/4 = 0.004\ s^{-1}$ per subunit) observed in the sulfite system is nearly 2 times as high as in the reductase system, suggesting that sulfite transfers electrons more efficiently to Hb than does P-450 reductase.

While the details of this reaction process have yet to be determined and the range of possible substrates is not yet known, the combination of sulfite and hemoglobin appears to be an efficient method to be explored for the convenient bioactivation of C–H bonds.

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(12) Segel, I. H. *Enzyme Kinetics*; John Wiley & Sons: New York, 1975.
(13) Perez-Benito, J. F.; Arias, C. *Collect. Czech. Chem. Commun.* **1991**, *56*, 1552.