Kinetic Solvent Isotope Effects during Oxygen Activation by Cytochrome P-450cam

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Received October 20, 1993

In spite of extensive investigation, the chemical and biochemical mechanisms of dioxygen activation by the superfamily of enzymes known as cytochromes P-450 are not completely understood. The actual steps of oxygen bond dissociation and product formation in the catalytic cycle have been characterized only by indirect studies of model compounds,[1] substrate analogs,[2] and molecular modeling.[3,4] Models for oxygen activation have also been proposed based on site directed mutagenesis.[5,6] Cytochrome P-450cam is one of the most extensively investigated enzymes in this family of heme monooxygenases and catalyzes the stereo- and regioselective hydroxylation of camphor. The electrons for this reaction are sequentially transferred from the electron-transfer protein, putidaredoxin, which in turn is coupled to the NADH-dependent flavoprotein, putidaredoxin reductase, as the ultimate source of reducing equivalents. We began an investigation of oxygen activation in cytochrome P450cam to answer some of the questions posed by differences in the various proposed models. Kinetic solvent isotope effect (KSIE)[7,8] methodology is applied, utilizing deuterium oxide in place of protium oxide, to dissect the mechanism of oxygen activation and determine the source and involvement of protons in the reaction.

The study of KSIE in cytochrome P-450cam requires a kinetic study of the catalytic cycle[5] beginning with substrate binding and proceeding through first electron transfer, oxygen binding, and second electron transfer, in order to identify steps isotopically sensitive to solvent substitution (D_2O for H_2O). Kinetic experiments in this last step are not able to separate second electron transfer from the extremely rapid substrate hydroxylation steps following the rate-limiting electron transfer from putidaredoxin. KSIE studies reveal that second electron transfer leading to O–O bond scission is the only step of the catalytic cycle displaying a significant solvent isotope effect of 1.8 ± 0.05 in single-turnover experiments.[9] The isotope effect observed is important since it links proton transfer to the unusually slow electron transfer from putidaredoxin (Pd) to P-450cam[10] and subsequent commitment to catalytic steps. KSIEs for earlier steps in the reaction cycle involving substrate binding (0.92 ± 0.1), electron transfer (1.03 ± 0.04), and dioxygen ligation (1.00 ± 0.03) are not sensitive to isotopic substitution of the solvent and also argue that the observed KSIE in second electron transfer is not the result of an isotope effect in P-450/Pd binding. Rates for all reactions were measured in samples incubated in deuterated buffer as well as diluted into protium buffer to confirm that slow exchange of hydrogenic sites within the protein were not responsible for the observed results.

A more detailed study of KSIEs was performed by using a proton inventory method[8] to determine the mechanism of proton involvement in oxygen activation. Figure 1 shows the nonlinear polynomial fit to the measured KSIE as a function of the percentage D_2O in the reaction mixture. The curvature of the fitted line is consistent with a minimum of two protons involved in the transition state of the reaction and, taken in concert with the magnitude of the KSIE, provides an opportunity to develop

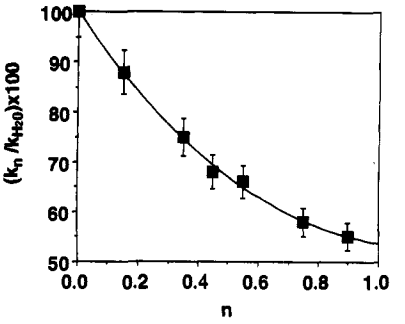

Figure 1. Proton inventory of cytochrome P-450cam second electron transfer and oxygen activation. Rates were normalized relative to the rates of second electron transfer through product release measured in H_2O. All data points represent at least three independent determinations of stopped-flow spectrophotometric experiments[9] between dioxygen-complexed P-450cam (2 μM) and reduced putidaredoxin (20 μM) with 2.5 mM metyrapone to stop reactions following single turnover. Reactions were maintained at 8 °C in D_2O/H_2O buffer mixtures listed as mole fraction of D_2O (n).

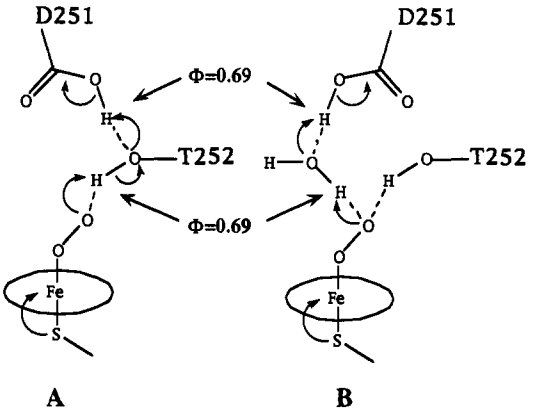

Figure 2. The charge relay model for the mechanism of oxygen activation in cytochrome P-450cam. (A) Mechanistic model of oxygen activation involving charge relay and proton delivery through T252 yielding a KSIE of 2.1. (B) An alternative model in which protons delivered to the distal oxygen are derived from an active site water molecule (KSIE = 2.1).

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(9) All reactions were carried out according to modifications to procedures described in ref 13: buffers contained 50 mM potassium phosphate as weighed mixtures of K_2HPO_4 and KH_2PO_4, pL of 7.4, and stock solutions of camphor, cytochrome P450cam, and putidaredoxin were prepared and equilibrated in the appropriate buffer solutions (H_2O or D_2O) before use. Proteins were reduced by treatment with $Na_2S_2O_8$ and passed over a Sephadex G25 resin or by catalytic quantities of putidaredoxin reductase and a 5-fold excess of NADH. In certain instances, solutions were made anaerobic with an oxygen scavenging system[14] or by exhaustive bubbling with prepurified argon. Stopped-flow spectrophotometry was carried out at 20 °C unless otherwise stated. Data from kinetic runs were analyzed by IGOR to yield pseudo-first-order rate constants, k_{obs} which were used for subsequent data analysis.

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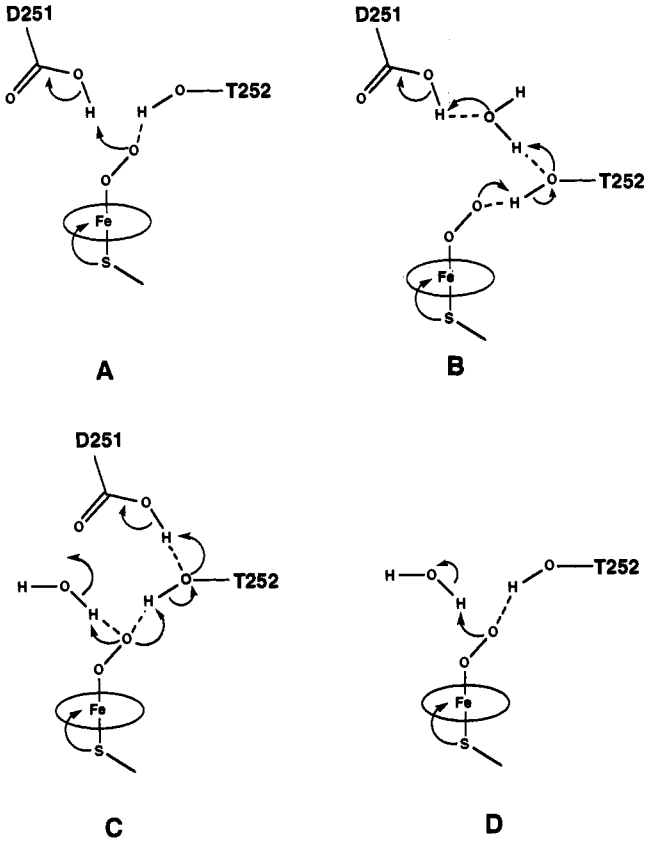
Scheme 1. Alternative Models for the Mechanism of Oxygen Activation in Cytochrome P-450cam Refuted by Kinetic Solvent Isotope Effects[a]



[a] (A) Direct single proton transfer to the distal oxygen from D251 (KSIE = 1.0). (B) Proton transfer to the distal oxygen mediated by a bridging water molecule and the hydroxyl group of T252 (KSIE = 3.0). (C) Protons transferred to the distal oxygen from D251 via bridging T252 hydroxyl and an active site water molecule (KSIE = 4.2). (D) Single proton transfer to the distal oxygen from an active site water molecule (KSIE = 2.0).

a mechanistic hypothesis of oxygen activation. Equation 1 utilizes the expected functional group fractionation factors taken from the literature[8] to calculate the expected magnitude of the KSIE for different models of oxygen activation proposed to involve multiple protons.

$$k_H/k_D = \prod \Phi_{\text{reactants}} \Big/ \prod \Phi_{\text{transition state}} \qquad (1)$$

A recently[5,11] proposed model suggests the involvement of a charge relay between the reduced oxygenated complex and a hydrogen bond network composed of the T252 hydroxyl and the D251 carboxyl, Figure 2A. The model is consistent with both the magnitude of the KSIE and the shape of the proton inventory curve. The charge relay model argues that, as second electron transfer occurs, charge density builds on the bound distal oxygen in the complex which is stabilized by a hydrogen-bonding interaction with T252. In the transition state, the T252-peroxy hydrogen-bonded complex receives a proton from D251 and at the same time transfers the hydrogen bonded proton to the reduced oxy complex. In this treatment, T252 can be thought of as a protonated oxonium-like species which, during proton transfer, would be expected to yield an overall isotope effect of approximately 2.1 given a fractionation factor of 0.69[12] both protons. An alternative indistinguishable mechanism may be operating which involves proton delivery through a bridging water molecule as shown in Figure 2B. In this model, D251 acts as described above but through the water molecule while T252 acts only to stabilize the building charge on the distal oxygen through a hydrogen-bonded interaction. KSIEs can eliminate other possible mechanisms that involve direct protonation of the bound peroxy complex with D251, or via different water mediated processes as shown in Scheme 1, since calculated KSIEs predict that these mechanistic models would yield KSIEs of unity or significantly different from the experimental value observed.

In summary, KSIEs have been used to probe the cytochrome P-450cam reaction cycle from a proton's point of view and are consistent with a charge relay hypothesis for oxygen activation. Experiments are ongoing to further explore the involvement of proton and solvent in the cytochrome P-450 reaction cycle.

Acknowledgment. The authors wish to thank Dr. Nancy Gerber and Ron Hernan for insightful discussions during the preparation of this paper. This work was supported by Grant GM 31756 from the National Institutes of Health.