did not affect the catalyst activity. Reactions were carried out for 24 h in order to collect a sufficient quantity of gasoline and diesel range products for the GC–CIMS product analysis. The C_5–C_{16} hydrocarbons and oxygenates were analyzed on a Hewlett-Packard (Model 5280) gas chromatograph equipped with an OV-101 capillary column. A VG Analytical mass spectrometer coupled to the GC was employed in the product identification. Isobutane was the reagent gas in the CIMS analysis.

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Registry No. CO, 630-08-0; potassium, 7440-09-7; iron, 7439-89-6.

A Conserved Residue of Cytochrome P-450 Is Involved in Heme-Oxygen Stability and Activation

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The cytochrome P-450 monooxygenase systems have received substantial attention due to their unique spectral and chemical properties.[1,2] Of particular interest are the precise chemical mechanisms involved in the binding and activation of atmospheric dioxygen and the subsequent functionalization of an unactivated carbon substrate.[3,4] We now report the first demonstration of a particular active site feature of cytochrome P-450 that is essential for efficient reduction and activation of molecular dioxygen, yet is independent of the substrate binding and spin-state equilibria processes. This has been accomplished by site-directed mutagenesis of threonine 252, a residue conserved among all known P-450 sequences, to an alanine (T252A),[5] in the active site of the *Pseudomonas* cytochrome $P\text{-}450_{cam}$.

Although the individual cytochrome P-450 isozymes demonstrate marked differences in their substrate specificity,[6] predictions based on amino acid sequence analysis indicate that they share several conserved structural features.[7-9] In particular, a long proximal α-helix exhibits strong homology throughout a diverse cross section of the P-450s. The high-resolution X-ray crystal structure of cytochrome $P\text{-}450_{cam}$[10] and molecular modeling studies[10-13] suggest that the juxtaposition of oxygen and substrate is controlled by specific residues in this helix. Specifically, the I-helix contains a region of local deformation of the normal helical structure arising from a direct hydrogen bond between the peptidyl carbonyl of G248 to the side-chain hydroxyl group of T252 rather than the expected peptidyl nitrogen of the threonine residue. This unique hydrogen bond, combined with other hydrogen bonds and ion-pair interactions, produces a "kink" in the helix directly adjacent to the active site and heme iron. In addition, this defined structure contains a hydrogen-bonding network which results in the presence of protein-sequestered, ordered water molecules directly adjacent to the oxygen binding pocket. This network of hydrogen bonds may conceivably provide a source of protons to stabilize a negative charge associated with the various reduced states of oxygenated P-450.[10]

Our mutation of the conserved threonine residue to an alanine yields a stable holoprotein which is expressed in *Escherichia coli*.[14,15] The purified protein displays optical and ferric EPR

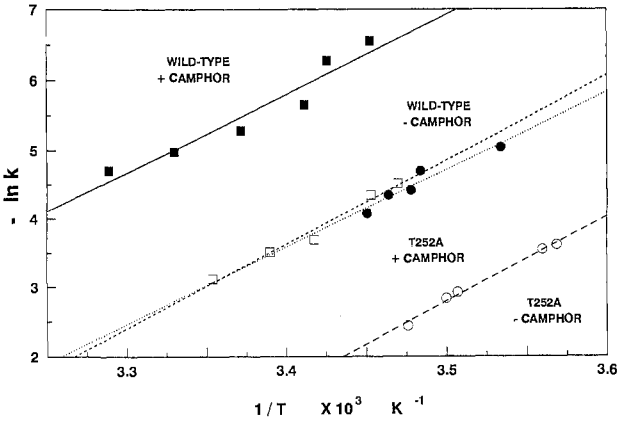

Figure 1. Comparison of substrate-free and -bound autoxidation rates of T252A and wild-type $P\text{-}450_{cam}$. Data points are denoted as follows: ■, substrate-bound wild-type; □, substrate-free wild-type; ●, substrate-bound T252A; ○, substrate-free T252A. Reactions were conducted in 700 μL of 50 mM Tris buffer, pH 7.4, 150 mM KCl, 4 mM EDTA, 0.5 μM proflavin, and 15 μM wild-type or T252A mutant. Camphor was 800 μM in the substrate-bound states. Autoxidation rates, k (s^{-1}), were measured as described.[10]

Table I. Stoichiometry of Camphor Metabolism

	wild-type (%)	T252A (%)
NADH consumption[a]	100 ± 1	100
5-*exo*-hydroxycamphor[b]	100 ± 1	5 ± 2
H_2O_2[c]	0	51 ± 2
"oxidase" water production[c]	0	22
5-*exo*-hydroxycamphor from H_2O_2-supported hydroxylation[d]	100 ± 1	16 ± 3

[a] NADH consumption was measured at 25 °C by the decrease in absorbance at 340 nm. Reaction mixtures contained 0.35 μM $P\text{-}450_{cam}$ or T252A, 2.4 μM putidaredoxin reductase, 10.6 μM putidaredoxin, 600 nmol of NADH, 150 mM KCl, 50 mM Tris, pH 7.4, and 600 nmol of camphor in a total volume of 500 μL. [b] A 500-μL reaction volume containing 50 mM Tris, pH 7.4, 150 mM KCl, 1 μmol of camphor, 4.4 μM putidaredoxin, 2 μM putidaredoxin reductase, 1 μM $P\text{-}450_{cam}$ or T252A, and 500 nmol of NADH was incubated for 15 min; 10 nmol of 3-*endo*-bromocamphor was added as an internal standard, and the solution was extracted with 1.5 mL of CH_2Cl_2, concentrated, and analyzed by gas chromatography as described.[15,26] [c] For experimental details, see: Atkins, W. M.; Sligar, S. G. *Biochemistry* **1988**, *27*, 1610-1616. [d] A reaction containing 20 μM $P\text{-}450_{cam}$ or T252A, 1 mM camphor, 50 μmol of H_2O_2, and 50 mM KP_i, pH 7.0 was halted after 10 min by the addition of $NaHSO_3$. Extraction of product and analysis were carried out as described.[15,26]

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spectra that are identical with those of the wild-type protein.[16-18] The addition of camphor to the mutant protein induces a low- to high-spin transition of the ferric center analogous to the native cytochrome P-450$_{cam}$. The dissociation constant for the substrate camphor to T252A was found to be 0.85 μM (25 °C), essentially equivalent to the wild-type value of 0.57 μM.[19,20] NADH consumption rates, and the production of 5-*exo*-hydroxycamphor as a sole product by the wild-type and mutant, also suggest that the integral protein conformation is conserved. In contrast, autoxidation of the ferrous, oxygenated species was drastically altered (Figure 1). The presence of substrate[23] and the structural features induced by threonine at position 252 stabilizes the one-electron-reduced, dioxygen-bound P-450 species (Figure 1). Similar activation energies for autoxidation suggest that camphor stabilizes the wild-type and the T252 mutant to the same extent and that variations in pre-exponential factors are primarily responsible for the 10-fold increase in autoxidation observed with T252A. Thus, as suggested by Poulos, T252 appears to be involved in stabilizing the oxygen complex.[10]

Although one-electron autoxidation is greatly enhanced, it cannot solely account for the fate of the NADH-derived reducing equivalents in the mutant protein, as the rate of NADH-coupled electron transfer into cytochrome P-450 is significantly faster than the autoxidation rate in both the wild-type and the mutant.[24,25] Stoichiometry measurements of T252A show a 5% product yield relative to the wild-type, with 51% of the NADH reducing equivalents appearing as hydrogen peroxide and 44% as excess water formation by a four-electron reduction of oxygen (Table I).[26-28] This is the first demonstration of a mutation in the cytochrome P-450s which alters the branching ratio of oxygenase and oxidase activities. In addition, the turnover of camphor by the exogenous oxidant hydrogen peroxide[29-32] is also dramatically reduced. Assuming that the binding and stabilization of hydrogen peroxide is similar to that of dioxygen, the decrease in product yield may be attributed to a disruption in a common mechanism of oxygenase and peroxygenase catalysis. These results demonstrate a crucial role of T252 in P-450 catalysis. X-ray crystallography should provide insight as to whether these effects are due to perturbation of the kinked I-helix, specific chemical alterations of the hydrogen-bonding environment, or availability of protons to aid in dioxygen bond cleavage.

Note Added in Proof. During the proof stage of this manuscript, an independent investigation reporting results with the mutation of threonine 252 was published (Imai, M.; Shimada, M.; Watanabe, Y.; Matsushima-Hibiya, Y.; Makino, R.; Koga, H.; Noriuchi, T.; Ishimura, Y. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 7823–7827). These authors, however, did not consider the possibility of excess water production by the *oxidase* activity of P-450$_{cam}$, and hence did not completely determine the effect of this mutation on the overall stoichiometry.

Acknowledgment. This research was supported by National Institutes of Health Grants GM31756, GM33775, and RRO1811.

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The Dehydrophenyl Anion and the Gas Phase Ion Chemistry of Benzyne

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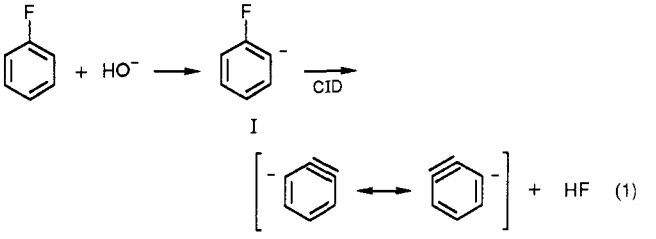
We report that in the gas phase the fluorophenyl anion (I) readily undergoes collision-induced dissociation (CID) to form an ion, $C_6H_3^-$, which, on the basis of its chemical reactions, we formulate as the 2,3-dehydrophenyl anion (II). Judging from its relatively weak basicity, II is a highly delocalized species which can be used to investigate the gas phase ion chemistry of its conjugate acid, benzyne, whose reactions closely parallel those in solution.[1]

For the preparation of II we used our tandem flowing afterglow–selected ion flow tube (FA–SIFT).[2] In the source flow tube we generated the fluorophenyl anion (I) by proton abstraction from fluorobenzene. This ion was mass-selected by the SIFT quadrupole and injected into the second flow tube. At low injection potentials, anion I survives intact, but at higher potentials (ca. 50 V),[3] it is completely converted to II by collision-induced dissociation in the helium buffer gas present at 0.5 Torr in the flow tube (eq 1).[4]



I

II

An exact measurement of the gas-phase basicity of II is complicated by an apparent kinetic barrier to its protonation as well as by the high reactivity of the benzyne, which is formed once protonation has occurred. For example, when II is allowed to react with CH_3OD, methoxide ion formation is observed in competition with up to three exchanges of hydrogen for deuterium; the overall efficiency of the reaction is 0.3–0.4. II does not abstract a proton from acids weaker than methanol ($\Delta G°_{acid}$ = 374 kcal mol^{-1}),[5] nor does it exchange with D_2O. We therefore estimate

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